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THE ORIGIN AND FUNCTION OF OPSONINS IN EXPERIMENTAL BRUCELLOSIS OF GUINEA PIGS

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Studies on blood opsonins in human brucellosis and in individuals with previous contact with brucella demonstrated great differences in opsonin titer of plasma. Although healthy individuals free of contact had insignificant quantities of specific plasma opsonin, subjects who had had contact or were ill with the disease showed increases in some instances of more than ten million opsonin units.[1] In general, the increase in opsonin titer paralleled the severity of illness but bore no quantitative relation to agglutinin titers. Because of obvious limitations in obtaining comparable morphological and clinical data, the significance of plasma opsonins has been sought by correlating plasma opsonins with pathological changes of brucellosis in guinea pigs. The relationship between the extent and intensity of brucellosis lesions and the plasma opsonin titer suggested that the lesion might be the site of formation of opsonins for brucella. Therefore, the experiments were extended to ascertain this point.

METHODS AND MATERIALS

Guinea pigs weighing 200 to 250 g were of the Hartley strain.

Brucella suis PSIII, a smooth virulent strain, grown on tryptose agar slants, was suspended to a concentration of 1×10^{10}/ml in saline solution. For the opsonin studies, they were killed by treatment with 1% formaldehyde for 24 hours at 37 C. The formaldehyde was removed by washing twice and then resuspending the organisms in Krebs-gelatin solution[1] to the original concentration of 1×10^{10}/ml. For inoculation, the live organisms were suspended in tryptose saline solution. Cultures of blood or tissues were grown on fortified Bacto-tryptose agar at 37 C.

"Heparin" Lederle was used, 1 mg per 10 ml of blood.

Determination of opsonin titer of plasma.—Details of the method have been described.[1] An opsonin unit is that quantity of opsonin in 0.1 ml serum, heparinized plasma or tissue fluid which causes phagocytosis of *Br. suis* PSIII by 94 to 100% of polymorphonuclear neutrophils under standard conditions. The opsonin titer represents the number of opsonin units in 0.1 ml serum, heparinized plasma or tissue fluid, determined by dilution.

The test was carried out by mixing 0.1 ml of heparinized blood, containing 1 mg of heparin in 10 ml of blood, with 0.1 ml of Krebs gelatin solution containing 1×10^{9} formalin-killed *Br. suis* PSIII. The mixture was rotated 24 to 36 times a minute for 30 minutes at 37 C. Smears on glass slides, prepared at this time, were stained with Jenner-Giemsa stain. The opsonin titer was determined as follows: Blood cells which previously were washed 6 times, each time with 10-fold volumes of Krebs-gelatin solution, were mixed with equal volumes of various dilutions of plasma in Krebs-gelatin solution. This mixture of washed blood cells and diluted plasma was treated with the suspension of *Br. suis* PSIII as noted above. When mixtures containing gelatin were employed, the glass slides were warmed to facilitate preparation of smears.

Opsonin titer of tissue fluid.—Tissues of guinea pigs exsanguinated by cardiac puncture were rapidly weighed on a Roller-Smith torsion balance. About 0.05 to 0.2 g of tissue was ground with 0.9 its weight of distilled water in a small Potter homogenizer with an inside diameter measuring 1.0 cm. The liquid was then made isotonic by adding 9.0% sodium chloride in a volume equivalent to 0.1 tissue weight. Accurate manipulations of small volumes of liquid and homogenate of infected tissues were facilitated by using a mechanical pipettor designed and con-

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Guinea pigs weighing 200 to 250 g were of the Hartley strain.

1. Victor, J., Pollack, A. D., Raymond, R. and Valliant, J. 1952, J.A.M.A. **149**:807.

structed by Mr. Milton Frank. To 0.1 ml of the homogenized tissue in 0.9% sodium chloride was added 0.1 ml of the donor's blood cells which had been separated from heparinized plasma and washed 6 times in Krebs-gelatin solution. Various dilutions of the tissue fluid were prepared by addition of Krebs-gelatin solution. To 0.1 ml of the mixtures of tissue homogenate and blood cells

TABLE 1.—*Relative volumes of components of experimental and control procedures.*

	Tissue	0.9% NaCl	Blood cells	Bacteria suspension
Experimental	1	1	2	4
Control		2	2	4

was added 0.1 ml 0.9% sodium chloride containing 1×10^9 formalin-killed *Br. suis* PSIII. Treatment of these mixtures resembled that outlined for determination of opsonin titer of plasma. After smears were made, small portions of the original mixture of tissue, blood, liquid, and bacteria were centrifuged in graduated capillaries. Calibrated capillaries of blood counting pipettes, sealed by heavy rubber bands at both ends, were suitable for this purpose. The relative proportions of solid and liquid were measured after centrifuging 10 to 20 minutes at 3000 r.p.m. to a constant sediment volume. Since a control mixture was always set up for determining the phagocytic activity of washed blood cells free of plasma or tissue liquid, the relative proportions of the blood cells and liquids free of tissue cells were determined as already outlined. The difference between the proportions of solid and liquid in the tissue and control mixtures was due to tissue solids. The percentage of tissue liquid in the homogenate was calculated from the percentage of tissue solid. The ratio of the volume of tissue liquid to the saline which was added could also be calculated.

The proportions of the various components of the experimental and control procedures are outlined in table 1. Calculations were made as follows:

a = percentage of tissue and blood cell solids

$$= \frac{\text{volume of tissue and blood cell solids} \times 100}{\text{total volume}}$$

b = percentage of blood cell solids

$$= \frac{\text{volume of blood cells solids} \times 100}{\text{total volume}}$$

c = percentage of tissue which was solid $= 8(a-b)$

d = percentage of tissue liquid $= 100 - c$

e = dilution of tissue liquid $= \frac{d}{100+d}$

RESULTS

Changes in blood opsonin titer during brucellosis in guinea pigs.—Guinea pigs were injected intradermally over the thorax in the right axillary line with 52 *Br. suis* PSIII, determined from plate counts. At intervals thereafter, 2 animals were exsanguinated by cardiac puncture through the thorax. Cultures were made from blood, the regional right axillary and the distant inguinal, cervical, left axillary or other lymph nodes as indicated by the presence of gross lesions, and from liver and spleen. Opsonin titers were determined in heparinized cardiac blood. The tissues which were examined microscopically included heart, aorta, thymus, lung, spleen, right axillary lymph node (referred to as the regional lymph node), distant lymph nodes (usually left inguinal), liver, pancreas, adrenal, kidney, reproductive organs, thyroid, parathyroid, brain, pituitary, femoral bone marrow and skeletal muscle. Opsonin titers were determined also in cardiac blood of 25 normal guinea pigs. The normal titers were less than 1, with percentages of neutrophils phagocytizing brucellae ranging from 7 to 32, with a mean of 19. The blood opsonin titers of nineteen guinea pigs with brucellosis at various periods of infection are recorded in table 2. Also recorded are the results of the culture of the tissues and blood for brucella and the essential morphological changes in the organs. Increases in blood opsonin were evident first at 2 weeks, when the percentage of neutrophils containing brucellae was 89 and 95, respectively. For the following 20 weeks, the opsonin titers were elevated in all except one guinea pig with a normal opsonin content 8 weeks after injection of *Br. suis* and no specific lesions of brucellosis, although brucella was cultured only from the liver containing a few foci of necrotic liver cells.

The characteristic lesion of *Br. suis* infection was a granuloma consisting of epithelioid cells concentrically arranged in masses which often coalesced and underwent central necrosis. The lesions occurred in all lymph nodes and spleen, leading to great enlargement of these organs, in liver, renal pelvis, epididymis, myometrium, and bone marrow, and occasionally in lung, heart, thyroid and scesses, the latter showing specific granulomatous changes in their walls.

There was little correlation between recovery of brucella and the occurrence of specific lesions after 15 weeks, or between blood opsonin levels and the duration of brucellosis. However, a correlation was maintained between blood opsonin titer and the intensity and distribution of specific lesions. Experi-

TABLE 2.—*Opsonin titers, cultures and lesions of brucellosis in 19 guinea pigs. Only animals with lesions have elevated opsonin titers.*

Opsonin			Brucella culture					Lesions									
Weeks of Infection	Titer	Percent phagocytosis	RALN	LILN	Spleen	Liver	Blood	Heart	Lung	Spleen	RALN	LILN	Liver	Renal pelvis	Epididymis	Uterus	Marrow	Meninges
0	0	7–32																
1	0	24	+		0	0	0				1							
1	0	19	+	0	0	0	0											
2	0	89	0	0	+	0	0				1							
2	0	95	+	+	+	+	0			1	1		1				2	
3	10		+	+	+	+	0			2	2	1					2	
3	1		+	+	+	+	+	1	1	1	2	1	1	2		1	2	
4	10^2		+	+	+	+	0		1	1	3	2	3	2	1		2	2
4	10		+	+	+	+	+		1	2	3	2	2	2		1	2	2
5			+	+	+	+	+	2	1	3	3	2	3	2		2		2
5			+	+	+	+	+		1	3	3	3	3	2	2			
6	10		+	+	+	+	+			3	4	3	2			2	2	
6	10		+	+	+	+	0			3	4	3	2	2			2	
8	10		+	0	+	0	0			3	4	2	2	2	3			
8	0	33	0	0	0	+	0						FN					
10	10^2		+	+	+	0	0		2	4	4	2	2	1	2			
10	1		+	+	+	0	0		2	2	2A	2	2					
15	0	78	0	0	0	0	0	BE			2	CA						
15	1		0	0	0	0	0			2A		PA		2	3A			
20	1		0	0	0	0	0			A	1	2A	A		2A			

Lymph nodes: RALN, right axillary; LILN, left inguinal.
+, positive culture; 0, negative culture.
Lesions: 1, reticulum cell hyperplasia or epithelioid infiltration.
2, reticulum cells plus granuloma.
3, reticulum cells plus necrosis.
4, reticulum cells plus necrosis plus abscess.
A, abscess; CA, cervical abscess, positive culture; FN, focal necrosis; PA, peritoneal abscess, positive culture; BE, bacterial endocarditis.

meninges. At different periods of infection certain organs became free of lesions while others acquired them. Abscesses were abundant from 6 weeks on.

Although lesions persisted throughout the 20 weeks of study, the regional lymph nodes, spleen, liver, blood and most distant lymph nodes were sterile when cultured after 15 weeks. At this time positive cultures were obtained from remote sites such as cervical lymph nodes or retroperitoneal abments were therefore undertaken to determine where opsonin was most abundant by measuring the opsonin content of blood and organs of guinea pigs with brucellosis.

Comparison of the opsonin contents of blood and different tissues.—Intradermal injection with 600 *Br. suis* PSIII was made over the thorax in the right midaxillary line. At intervals thereafter, tissues regularly showing specific lesions were examined, i.e., the site of injection, the regional and distant lymph nodes,

liver, spleen, epididymis, lung and bone marrow. Adrenal and kidney were also examined because lesions rarely occurred in these organs although brucellae were readily cultured from them at the time of the observations.

The results of these studies are recorded in table 3. Whenever blood opsonins were elevated, the opsonin level of the regional lymph node was elevated, and in one instance, at 12 days, node fluid and blood increased, the opsonins of these fluids were compared simultaneously. When the lesions were only in regional lymph nodes, the blood opsonin might be derived from these tissues. Direct comparisons between that node and the blood would yield information about opsonin concentrations in them. Thus, possible accumulation in the blood of opsonin from other infected tissues would be avoided.

TABLE 3.—*Opsonins of blood and tissue fluids of guinea pigs infected subcutaneously with 600* Br. suis *PSIII. Only tissues with brucellosis lesions have elevated opsonin titers.*

Tissue	Days after infection							
	7		10		12		16	
	Dilution	Percent phagocytic neutrophils	Dilution	Percent phagocytic neutrophils	Dilution	Percent phagocytic neutrophils	Dilution	Percent phagocytic neutrophils
Blood	0	18	2.0	98	10	97	0	100
	∞	38	∞	69	100	29		
Regional L.N.	4.9	30	7.2	96	13	96	3.5	70
					130	59		
Distant L.N.	2.3	18	2.7	16	2.7	82	7.0	48
Liver	2.2	39	2.1	76	2.0	32	5.0	33
Spleen	4.5	36	3.7	62	3.1	98	6.0	36
Kidney	2.4	21	2.6	55	2.2	29	5.0	30
Adrenal	5.4	18	9.3	29	7.3	25	7.0	35
Lung	1.2	18	4.3	57	6.4	86	3.7	27
Testis	2.0	23	2.2	79	2.0	98	3.7	21
Skin	2.5	21	2.4	60	2.3	96		
Marrow	8.0	12	9.8	24	8.7	40	15.0	34

was higher than in the blood. In this same animal, the opsonin contents of fluids of spleen, lung, testis, distant lymph nodes and site of injection were also elevated although the titers were not determined. However, even in this guinea pig the opsonin content of the adrenal and kidney appeared unaltered. Although all tissues of guinea pigs infected for 10 to 16 days contained brucellae, as shown by blood culture, only tissues with lesions had increased opsonins. To determine whether opsonins were produced or absorbed by tissues with specific lesions, further experiments were performed.

Opsonin in plasma and regional and distant lymph nodes after intradermal injection of brucella.—Since opsonin concentrations of both regional lymph

Guinea pigs were infected as described above with 600 *Br. suis* PSIII. Because the regional lymph node was the first tissue with lesions (tables 2 and 3), the opsonin concentration was measured in blood and regional and distant lymph nodes, the latter including the pooled cervical, left axillary, and bilateral inguinal lymph nodes. Opsonin titers were measured by dilutions of plasma or tissue fluids. The results of 7 of 17 experiments are recorded in table 4. When comparing liquids, the opsonins would be highest in the one with highest percentage of neutrophils in the same dilution or the same values in higher dilutions. The highest opsonin values have been italicized. In only one of the experiments was the blood opsonin concentration higher than that of regional

lymph node fluid. Six of the 7 experiments showed lymph node opsonins higher than blood, in some instances twice as high. The results of 10 of the experiments are not recorded because the dilutions of the plasma or tissue fluid were such that no conclusions could be drawn about the relative concentrations of opsonin in the blood and regional lymph node fluids. In all 17 experiments. the opsonin was lowest in the distant normal lymph nodes. Whether the opsonin was concentrated from the blood or produced in the inflamed lymph node was investigated next.

TABLE 4.—*Opsonin titers of blood and of regional and distant lymph nodes of guinea pigs infected with* Br. suis *PSIII. Lymph nodes with specific lesions have highest opsonins.*

Exp. no.	Day of infection	Blood		Lymph node fluid			
				Regional		Distant	
		Plasma dilution	Percent phagocytic neutrophils	Dilution	Percent phagocytic neutrophils	Dilution	Percent phagocytic neutrophils
1	12	1 10 100	97 97 29	3 15 *150*	100 96 59	3	82
2	7	1 2 10	68 56 40	*10.6*	54		
3	10	2 4 *8*	100 100 *100*	3 6	97 71	3	28
4	14	1 2	92 46	*3*	50	3	28
5	14	1 2 4	100 90 76	*5.3*	80	3.1	29
6	14	1 3 6 12	100 100 96 66	10.6 *21.2*	92 74	3	24
7	15	6 12	96 89	11.4 *22.8*	98 90	12	21

Opsonin of blood and of normal and nonspecifically inflamed lymph nodes of guinea pigs with passibely transferred opsonins.—Several investigations[2–5] have demonstrated that agglutinins, neutralizing antibodies or antitoxins were not absorbed in nonspecifically inflamed tissues. To determine whether opsonin for brucella was absorbed by nonspecifically inflamed tissue, the following experiments were performed. Heparinized plasma with opsonin titers of 10^2 to 10^3 was obtained from guinea pigs infected 3 to 6 weeks previously. Sterility was ascertained by cultures of the plasma in tryptose broth for a week. Lymphadenitis was produced by intradermal injection of 1×10^8 *Streptococcus hemolyticus*, type C, or of 90 spores of *Bacillus anthracis* M 36 in 0.1 ml 0.9% sodium chloride. Comparison in 40 guinea pigs of doses of 1×10^2, 1×10^4, 1×10^6, 1×10^8 and 1×10^{10} *Str. hemolyticus* showed that 1×10^8 organisms pro-

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duced maximal regional lymph node hyperplasia, congestion and neutrophilic infiltration in 3 to 6 days, persisting 2 weeks after injection, without systemic dissemination of the organism or changes in other tissues. Intense lymphadenitis with congestion and edema, fibrin and neutrophilic exudation appeared within 24 hours after injection of *B. anthracis*. Guinea pigs with lymphadenitis were injected intraperitoneally with 1 ml of plasma with high brucella opsonin 1 day before sacrifice. The results are recorded in table 5. Although brucella opsonin increased equally in both normal and inflamed lymph nodes, the increase was slight as compared with that of plasma.

TABLE 5.—*Opsonins of blood and of normal and nonspecifically inflamed lymph nodes of guinea pigs with passively transferred opsonins. Elevated blood opsonins were not concentrated in fluid of inflamed nodes.*

Exp. no.	Infecting bacteria	Day of infection	Plasma		Lymph node fluid			
					Inflamed		Distal	
			Dilution	Percent phagocytic neutrophils	Dilution	Percent phagocytic neutrophils	Dilution	Percent phagocytic neutrophils
1	Hemolytic strep. type C	3	6	97	4.6	64	5.1	67
2	Hemolytic strep. type C	3	10 100	96 62	6.0	67	6.0	69
3	Hemolytic strep. type C	6	10 100	100 46	3.0	58	5.8	70
4	Hemolytic strep. type C	7	10 100	100 84	6.0	64	6.0	70
5	Hemolytic strep. type C	4	6 10	100 91	4.3	62	5.2	86
6	Hemolytic strep. type C	5	10	100	6.0	68		
7	Hemolytic strep. type C	7	10	90	6.0	52	6.0	48
8	Hemolytic strep. type C	8	10	72	6.0	48	6.0	45
9	Hemolytic strep. type C		10	68	3.7	27	3.6	33
10	*B. anthracis* M 36	2	6 10	100 91	6.0	67	6.0	58

Opsonin in blood and nonspecifically inflamed lymph nodes containing brucella in guinea pigs with passively transferred brucella opsonins.—It was possible that the presence of specific antigen, namely, brucellae, might affect the concentration of opsonins in the fluids of nonspecifically inflamed tissue. Guinea pigs were infected intradermally with 1×10^8 *Str. hemolyticus*, type C. After developing lymphadenitis in the regional axillary lymph nodes, 1×10^9 *Br. suis* PSIII in 0.1 ml saline was injected subcutaneously near the inflamed lymph nodes, and plasma with opsonin titer of 10^3 was injected intraperitoneally in a dose of 1 ml per 250 g guinea pig. The animals were sacrificed 24 hours later when in 6 control, normal guinea pigs the brucella count by the plate dilution method ranged from 10^5 to 5×10^7 per g of regional lymph node. This number of organisms was similar to that observed in lesions of brucellosis in guinea pigs. The results of the experiments are recorded in table 6. In spite of high blood opsonins in all animals, the brucella opsonins in the nonspecifically in-

flamed lymph nodes containing brucellae were not elevated, being no higher than those of normal lymph nodes.

DISCUSSION

The observations described above confirm and extend those of McMaster and Hudack,[2] McMaster and Kidd,[3] and Oakley, Warrack and Batty,[5] who found antibody formation due to bacterial vaccines, vaccinia and toxins, renon and Sullivan,[7] Seegal and Seegal,[8] and later Walsh and Cannon[9] demonstrated agglutinins in higher concentrations at the site of inoculation than in other tissue but did not exclude the possibility that inflammatory tissue absorbed antibodies from the blood. Burnet and Lush,[10] on the other hand, found antibody formation in the local lymph nodes but not in the skin of rabbits. More direct evidence for antibody for-

TABLE 6.—*Opsonin in blood and nonspecifically inflamed lymph nodes containing brucella in guinea pigs with passively transferred brucella opsonins. Elevated blood opsonins were not concentrated in fluid of inflamed lymph nodes.*

Exp. no.	Days of streptococcus infection	Blood plasma		Lymph node fluids			
				Inflamed		Normal	
		Dilution	Percent phagocytic neutrophils	Dilution	Percent phagocytic neutrophils	Dilution	Percent phagocytic neutrophils
1	3	6	100	2.5	27		
2	4	6	72	2.8	24		
3	5	10	100			2.2	76
4	6	10	100	2.3	52	3.8	43
5	6	10	100	3.8	58	10.0	39
6	7	10	100	2.5	60	5.5	56
7	7	10	100	2.9	73	6.0	56
8	8	10	100			2.9	48
9	9	10	100			2.1	45

spectively, mainly in the regional lymph nodes after intradermal injection and not in nonspecifically inflamed distant lymph nodes. The present experiments further demonstrated that antibodies were not even deposited in nonspecifically inflamed tissues containing live brucella antigen.

When brucellosis lesions developed in other tissues, such as distant lymph nodes, spleen, liver, kidney, lung, testis or the site of injection, opsonins also appeared in them. Smith, Orcutt and Little[6] found agglutinins to *Brucella abortus* in milk of one experimentally infected teat and not in the other three untreated teats of cows with *Str. hemolyticus* mastitis. The serum agglutinin for brucella increased later. Cannation in various organs has been described.[11–14]

The cells concerned in antibody formation are those usually ascribed to the reticulo-endothelial system. The fibroblast,[15] macrophage,[9,16–17] lympho-

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cyte,[18–23] neutrophil,[24] and more recently, plasma cell[25–28] have been implicated by much evidence. At different times the same authors have favored the lymphocyte or plasma cell.[18,28] The lesions of guinea pig brucellosis due to *Br. suis* consist of cells variously called epithelioid, endothelioid or reticulum cells, comprising more than 95% of the lesions' cell mass. The other cells are fibroblasts, lymphoid, mononuclear and plasma cells, and polymorphonuclear neutrophils, the latter increasing when necrosis occurred. The present studies, although strongly suggesting that the epithelioid cells were concerned in the formation of opsonins, offer no more definite data than that already presented for other cell types.

Employing antigens labeled with dye and radioactive sulphur, Garvey et al[29] interpreted high concentration of antigen in the kidney as evidence for the site of formation of antibodies. However, Kruse and McMaster[30] and McMaster and Kruse[31] have considered localization of labeled antigens in this light with great caution. Indeed, Dixon et al[32] suggest that dye-labeled radioactive antigen was localized differently from iodinated antigen.[33] In the present experiments, the presence of *Br. suis* in normal tissues, such as kidney and adrenal or in nonspecific lymphadenitis containing brucellae, was not associated with opsonins in those tissues unless specific lesions were also present. Guinea pigs with generalized brucellosis and high plasma opsonin titers, indicated in table 7, showed opsonins relatively low in kidneys free of lesions, and high in those with brucellosis pyelonephritis and in other tissues with specific lesions. With plasma opsonin titer of 1000, a renal opsonin titer of 5 was not unexpected, since the tissues were not

TABLE 7.—*Opsonin titers of plasma and tissue fluids in brucellosis.*

Days of infection	Plasma	Lymph nodes		Spleen	Liver	Kidney	Bone marrow
		Regional	Distant				
37	1000	150	90	150	150	5 a	150
40	100	15	15.6	15	15	15 b	15

a. Normal kidney.
b. Brucellosis pyelonephritis.

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washed free of blood. The opsonin titers in all tissues in late brucella infection were considerably lower than those in the plasma. These combined data suggest that even tissues with specific lesions of brucellosis did not freely absorb opsonin from the plasma. The higher opsonin in the plasma than in the individual organs at this time was probably due to greater release of opsonin from the lesions than disappearance of opsonin from the blood stream.

The experiments cited above have demonstrated that opsonins for brucella are produced in specific lesions of brucellosis. Neither the reticulo-endothelial system nor special organs need be implicated in this mechanism. X rays,[34,35] nitrogen mustards,[36] or hormones such as cortisone[37–42] or ACTH[38,43] decrease both tissue reactions and antibody formation. Results such as these add further support for the suggested relationship between specific lesions and opsonin formation. Whether other antibodies are similarly related to specific lesions remains to be established.

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SUMMARY AND CONCLUSIONS

1. The plasma opsonin titer of guinea pigs with brucellosis was related to the extent and intensity of specific lesions.

2. Fluid from tissues with brucellosis lesions showed increased opsonins which were higher than in plasma in early brucellosis. Later, opsonin in plasma was higher than in tissues.

3. Nonspecific inflammatory tissue in guinea pigs with passively transferred opsonins did not absorb opsonins even when the tissue contained brucellae.

4. The combined data indicated that brucella opsonins are formed in specific lesions of brucellosis.

EFFECT OF L-CYSTEINE HYDROCHLORIDE, DL-ALANINE, HYDROCHLORIC ACID AND SODIUM HYDROSULFIDE ON EXPERIMENTAL POLYARTHRITIS OF RATS

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Mouse and rat polyarthritis, induced by pleuropneumonia-like organisms (PPLO), represent a new but important type of disease. This disease has been studied by relatively few investigators. Collier[1] and Findlay, Mackenzie and MacCallum[2] found that gold compounds exert a preventive action against development of rat polyarthritis. Sabin and Warren[3] and Preston, Block and Freyberg[4] also reported that such compounds have a curative action on experimental mouse polyarthritis.

Although rodent polyarthritis is not identical in its pathology with human rheumatoid arthritis, certain similarities and the beneficial effect of gold in both suggested a common denominator in their etiology, and also the possibility that, through chemotherapeutic trials in animals, other agents could be found more effective and less toxic than the gold compounds. The common response to gold therapy further suggested that studies on animals might throw more light on the mechanism of action of these agents on human arthritis. Kuzell and associates[5–7] tested a number of compounds and found that cysteine hydrochloride, administered in 1% solution in drinking water, inhibits the development of the experimental disease in rats. They also reported[8] that cystine had a beneficial effect on rat polyarthritis. These findings are of interest because both cysteine and cystine are amino acids present in variable proportion in most proteins. They suggest that a diet rich in proteins, with high content of sulfur amino acids, might be important in increasing the resistance against development of arthritis. On the other hand, the same authors found that other SH (sulfhydryl) compounds, such as glutathione and BAL (British anti-lewisite), not only failed to prevent or reduce, but even considerably enhanced the incidence and severity of rat polyarthritis. It should be noted however, that these enhancing effects have been obtained with glutathione and BAL administered *parenterally*, whereas the inhibitory effect with cysteine was obtained by oral administration. It would thus seem evident that the inhibitory action of cysteine, as contrasted with the enhancing action of glutathione and BAL, could be due either to the route of administration or to the specific nature of the nonsulfhy-

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dryl radical of the amino acid or to both factors.

In order to test these possibilities, and to ascertain which part or parts of of the cysteine HCl molecule is responsible for the inhibitory action on the development of rat polyarthritis, we studied the effect of cysteine administered both orally and parenterally and also the effect of alanine and hydrochloric acid solutions in drinking water. The concentrations used were approximately equivalent to that of 1% L-cysteine HCl. The testing of alanine is suggested by the fact that this amino acid differs from cysteine only by its lack of an SH group in β position, and also because alanine is one of the products formed by the action of cysteine desulfurase on cysteine in the liver,[9] as expressed in the following equation:

$$\underset{\text{cysteine}}{HSCH_2—CHNH_2—COOH} \xrightarrow{H_2} CH_3—CHNH_2—COOH + H_2S$$
$$\text{alanine}$$

We also studied the effect of sodium hydrosulfide (HSNa) administered parenterally and the effect of dosage and early administration of oral cysteine HCl prior to the infection with pleuropneumonia-like organisms, as will be noted later.

EXPERIMENTAL

Cultures.—The L4 strain of pleuropneumonia-like organisms used was originally isolated by Preston and studied by Kuzell and associates in their work on experimental rat polyarthritis. Following Dr. Kuzell's procedures, the culture was maintained on a medium composed of: 3 g Difco dehydrated yeast extract, 7 g Difco nutrient broth concentrate, 10 g tryptose phosphate broth concentrate, and 2 g dextrose C. P. in one liter distilled water. For liquid medium 0.15 g of agar and for solid medium 20 g of agar were added. The mediums were adjusted to pH 8 before autoclaving, and 20% horse serum was added after cooling. On receipt, the culture was inoculated into rats and the organisms were recovered from the affected joints on two separate occasions. A greater incidence of polyarthritis is believed to result when several isolations of the same strain are combined just prior to infection. Thus, 3 separate cultures were made available for rat inoculation. Since the cultures grew luxuriantly on solid medium, it was possible to scrape off sufficient material to inoculate tubes containing 5 cc of broth for each culture. After 48 hours of incubation at 37 C the entire amount of each tube was transferred into Erlenmeyer flasks containing the amount of medium needed for each experiment. All cultures were incubated for 24 hours. Then transfers of 10 cc were made to fresh flasks, which were incubated for an additional 24 hours. We thus had available 2 sets of flasks which had been incubated for 24 and 48 hours, respectively. These were then pooled to supply the rat inoculum. Each culture was streaked on solid medium, and direct smears were prepared and examined before rat inoculation. Equal numbers of male and female albino rats were used. They were inoculated intraperitoneally with 3 cc of combined broth cultures.

Arthrogram score.—The average arthrogram score was calculated according to the scheme proposed by Sabin and Warren and modified by Kuzell et al. A numerical value of 5 was given for each hind leg which developed arthritis and a value of 4 for each front leg. The total number of points were added for all rats in the group and the average score determined by dividing the result by the number of rats in each group regardless of the percent incidence or percent survival for the group.

Effect of L-cysteine hydrochloride in drinking water.—A 1% solution of L-cysteine HCl, freshly prepared each day, was administered ad libitum to the rats. It was observed that at least for two days the animals drank the solution with great reluctance, due probably to its high acidity. The rats which were fed 1% L-cysteine HCl solution, the administration of which started the same day as the inoculation with PPLO, showed only a small difference in the incidence of polyarthritis as compared with the infected control animals:

9. Smythe, W. A. 1942, J. Biol. Chem. **142**: 387.

whereas 7 out of 9 control rats (78%) developed polyarthritis, 5 out of the 9 cysteine HCl-fed animals (56%) developed the disease.

In order to accustom the animals to the treatment and thus increase the intake of L-cysteine HCl, feeding of the control animals. There was also a considerable delay in the onset of the symptoms in the L-cysteine HCl-treated rats that developed the disease.

Another group of 16 rats treated orally with 1% solution of L-cysteine HCl, starting 2 days before inoculation with

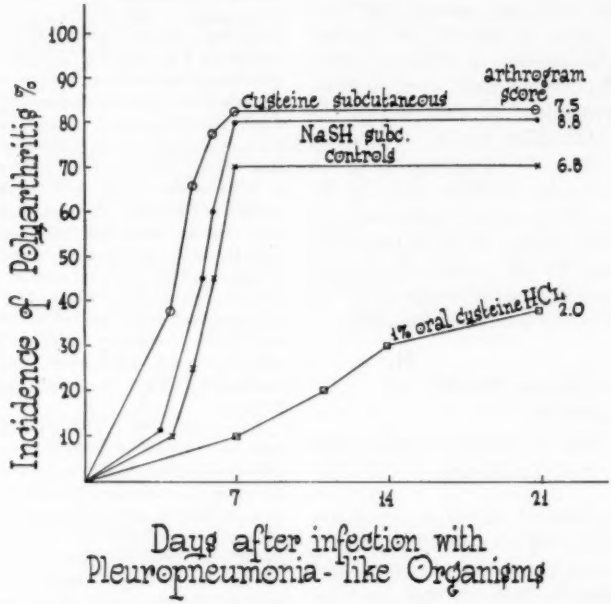

FIG. 1.—Effect of oral L-cysteine hydrochloride, parenteral L-cysteine and parenteral sodium hydrosulfide on the development of rat polyarthritis. Feeding of L-cysteine HCl started one week before inoculation with PPLO.

solution in one experiment was started 2 days and in another one week before inoculation with PPLO. Figure 1 shows the effect of 1% L-cysteine HCl solution administered one week before inoculation. While 9 out of 13 control animals (69%) developed polyarthritis, only 5 of the 13 cysteine HCl-fed rats (39%) developed the disease. Moreover, the severity of the disease was considerably less in those treated animals which did develop polyarthritis than in the PPLO, also showed a lower incidence of polyarthritis than the 16 controls. In this case however, no delay in the appearance of the symptoms was observed in those treated animals which developed the disease. This is shown in figure 2.

In order to test the effect of lower concentrations of L-cysteine HCl solutions administered over a longer period of time, 9 two-week-old rats were fed 0.1% solution of L-cysteine HCl for

4 weeks before inoculation with PPLO. These animals showed practically the same incidence of polyarthritis as the 9 control rats of the same age. Another experiment on 8 rats fed with 0.25% solution of L-cysteine HCl, starting three weeks before inoculation, showed a 38% incidence as compared with 66% incidence in 8 control rats.

cysteine HCl dissolved in 1 cc distilled water and neutralized with sodium bicarbonate were administered subcutaneously every day to rats inoculated with PPLO. Treatment was started the day of inoculation. In table 1 are given the results of 3 different experiments. Figure 1 shows the graph of experiment 2. These results show that

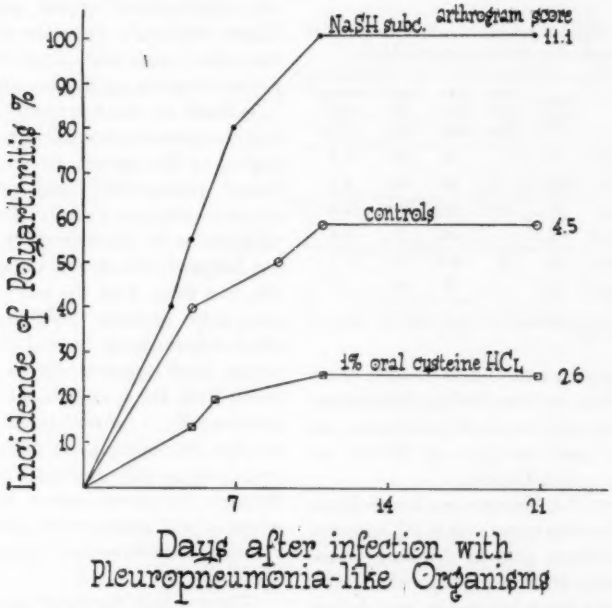

FIG. 2.—Effect of parenteral sodium hydrosulfide and oral L-cysteine hydrochloride on the development of rat polyarthritis. Feeding of L-cysteine HCl started two days before inoculation with PPLO.

The results with L-cysteine HCl administered in drinking water show that this amino acid exerts a considerable inhibitory action against development of PPLO-induced rat polyarthritis. This effect is more marked when treatment is administered earlier and when the concentration of the solution increases from 0.1 to 1%.

Effect of L-cysteine administered subcutaneously.—Doses of 20 mg of L-

TABLE 1.—*Effect of parenteral L-cysteine on the development of rat polyarthritis.*

Exp. no.		Number of rats	Number of injections	Incidence (percent)	Survival (percent)	Arthrogram score (avg.)
1	Treated	8	9	88	88	7.1
	Controls	9		66	100	6.6
2	Treated	10	12	80	100	7.5
	Controls	13		69	84	6.8
3	Treated	17	12	88	100	7.2
	Controls	17		35	100	3.5

cysteine administered subcutaneously exerts an enhancing effect on experimental polyarthritis similar to the effects reported for glutathione and BAL.

Effect of sodium hydrosulfide administered subcutaneously.—PPLO-infected rats were treated every day with 5 mg/kg sodium hydrosulfide in aqueous solution. Treatment was started the day of infection. Table 2 gives the results of three experiments. These results show that sodium hydrosulfide, administered subcutaneously, markedly enhances the incidence and severity of PPLO induced rat polyarthritis.

TABLE 2.—*Effect of parenteral sodium hydrosulfide on the development of rat polyarthritis.*

Exp. no.*		Number of rats	Number of injections	Incidence (percent)	Survival (percent)	Arthrogram score (avg.)
1	Treated	5	9	80	100	9.4
	Controls	9		66	100	6.6
2	Treated	10	8	80	100	8.8
	Controls	13		69	84	6.8
3	Treated	13	9	100	92	11.1
	Controls	16		56	100	4.5

* Graphs of experiments 2 and 3 are shown in figures 1 and 2, respectively.

Effect of D-L alanine and hydrochloric acid in drinking water.—A 0.2% solution of hydrochloric acid in drinking water was administered to a group of 17 rats, starting one week before the inoculation of the culture. This concentration corresponds approximately to the amount of hydrochloric acid contained in a 1% solution of L-cysteine hydrochloride. In the same experiment and starting the administration at the same time, another group of 17 rats was fed with a 1.5% solution of D-L alanine in drinking water. It was observed that the daily intake of hydrochloric acid solution was about the same as that of 1% solution of L-cysteine HCl. The intake of alanine solution was, on the other hand, somewhat higher.

Figure 3 shows that there is practically no difference between the incidence of polyarthritis in the two groups of treated rats and the control animals.

DISCUSSION

L-cysteine HCl, administered orally, obviously exerts an inhibitory action, while the same amino acid administered parenterally has an enhancing effect on the development of rat polyarthritis. These strikingly opposite effects with the same compound, given by different routes, deserve special attention.

It is not uncommon to find that chemical compounds exert certain pharmacological or therapeutic actions when injected parenterally and are inactive when administered orally. The behavior of cysteine in preventing or enhancing rat polyarthritis shows, presumably for the first time, that the same compound *can exert opposite therapeutic actions when administered by oral or parenteral routes.* Such opposite effects apparently result from the circumstance that, while parenterally administered cysteine reaches the general circulation rapidly, oral cysteine will previously be absorbed through the portal system in the liver, where it will undergo changes to which cysteine administered parenterally is not subjected.

Gilmore and Sprince[10] have demonstrated that SH compounds enhance the growth of human strains of pleuropneumonia-like organisms, and in our experiments we found that these compounds have a similar effect on the L4 strain of PPLO. The enhancing effect of parenteral cysteine and sodium hydrosulfide, as well as the effects of glutathione and BAL, on the development of rat polyarthritis can thus be explained by the role these compounds play in stimulating the growth of the

10. Gilmore, E. L. and Sprince, H. 1949, J. Bact. **57**: 473.

pathogenic organisms. On the other hand, cysteine administered orally is absorbed in the liver, where it is used in the synthesis of proteins or undergoes a process of desulfuration by the action of cysteine desulfurase.[9] Thus, the liver would act as a filter in preventing cysteine from reaching the general circulation and from supplying SH groups to the pleuropneumonia-like organisms.

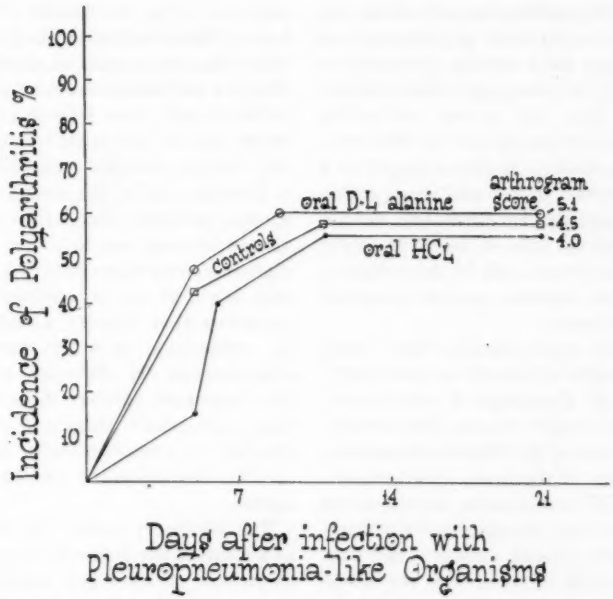

FIG. 3.—Effect of oral D-L alanine and oral hydrochloric acid on the development of rat polyarthritis.

These factors, however, do not explain why oral cysteine, retained in the liver, exerts an inhibitory action. If the liver acted only as a filter for the amino acid, we should expect oral cysteine to be, at most, therapeutically indifferent. The experimental results show, however, that cysteine administered orally not only does not enhance infection but plays an active part in protecting the animals against the disease.

By the action of cysteine desulfurase in the liver, cysteine produces hydrogen sulfide on one hand, and alanine and pyruvic acid on the other—a process which incidentally accounts for the glycogenic nature of cysteine. We found in our experiments that alanine is therapeutically inactive and that parenteral sodium hydrosulfide aggravates rather than inhibits the infection. The negative therapeutic results with alanine and hydrochloric acid show, however, that the inhibitory action of oral cysteine depends essentially on the presence of the SH group in the molecule.

The explanation of this apparent paradox might lie in the fact that parenterally administered sodium hydro-

sulfide and other SH compounds will circulate in the interstitial fluids outside the cells of the host whereas cysteine, administered orally, will generate hydrogen sulfide ***within*** the liver cells by the action of intracellular cysteine desulfurase.

We have no experimental data at the present time by the use of which we are able to formulate the mechanism whereby hydrogen sulfide, formed within the liver cells, might exert a protective action against polyarthritis. It could be postulated, however, as a working hypothesis, that the known activating effect of hydrogen sulfide on the intracellular proteolytic enzymes might be a factor involved in the inhibitory action of oral cysteine. Its formation within the phagocytic cells of the liver might play an important role in the enhancement of the defenses against invading microorganisms.[11]

It would seem possible that, while hydrogen sulfide formed intracellularly, following the absorption of oral cysteine in the liver, might activate the intracellular enzymes of the tissues and increase the defenses of the host, hydrogen sulfide and SH compounds, administered parenterally and circulating in the interstitial fluids, might activate the bacterial enzymes to which the are accessible and thus increase their pathogenicity, before these SH compounds are removed from the system by the tissues of the host.

The implication of these experimental results appear to be important. They show that a supplement of a specific amino acid in the diet can inhibit the development of an infectious disease. And they suggest that the protective effect of a high protein diet against infection may be due, in part, to one amino acid rather than to the entire protein molecule. They also now for the first time indicate that the same amino acid, protective when given orally, also exerts an enhancing effect in the same infection when administered parenterally. Our results also emphasize the fundamental role played by the liver in the defense against infectious diseases and suggest the importance of cysteine desulfurase in the mechanism of these defenses. They further indicate the possibility that resistance to diseases other than rat polyarthritis, including human arthritis and other infectious diseases, might also be increased by supplementary dietary administration of cysteine, or proteins rich in this amino acid. It is hardly probable that this particular disease in rats, due to a new and only slightly known organism (PPLO), is the only one that can be modified by such administration. Finally, a route is open for exploration of other amino acids. administered by different routes, on the resistance against different infections, particularly those not adequately affected by treatment with antibiotics or other bacteriostatic and bactericidal agents.

The inhibitory action of oral cysteine, as a result of the intracellular enzymatic formation of hydrogen sulfide, might also explain the effect of the gold salts in protecting the infected animals against polyarthritis. In a report on the toxicity and mode of action of the gold salts, one of the writers[12] pointed out that the gold compounds, through the formation of gold mercaptides, produce within the cell hydrogen sulfide, which in turn might be the active therapeutic factor in arthritis.

SUMMARY

1. Rats inoculated with the L4 strain

11. Perla, D. and Marmorston, J. 1941, Natural Resistance and Clinical Medicine, Boston, Little, Brown and Co.

12. Libenson, L. 1945, Exper. Med. & Surg. 3: 146.

of pleuropneumonia-like organisms were treated with oral and parenteral cysteine hydrochloride, parenteral sodium hydrosulfide, oral alanine and oral hydrochloric acid.

2. Whereas orally administered cysteine hydrochloride inhibited considerably the development of polyarthritis, parenteral cysteine enhanced the incidence and severity of the experimental infection.

3. Oral DL-alanine and hydrochloric acid in concentrations corresponding approximately to that of cysteine hydrochloride did not affect the course of the disease.

4. Sodium hydrosulfide administered parenterally enhanced the development of rat polyarthritis.

5. The implications of these results are discussed, and it is suggested that the observed inhibitory effect of oral cysteine on one hand, and the enhancing effect of the infection by parenteral cysteine on the other, might be due to the activation of the intracellular enzyme systems in the host and in the invading microorganism, respectively.

CHEMOTHERAPY OF EXPERIMENTAL INFECTIONS OF MICE WITH *SCHISTOSOMATIUM DOUTHITTI, SCHISTOSOMA MANSONI* AND *SCHISTOSOMA JAPONICUM*

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Of the three clinically important schistosomes, *Schistosoma mansoni* is the only species that has been maintained in the laboratory with ease for large screening programs. A drug effective at one dose level against one species of schistosoma may, however, be partially effective or ineffective against other species in the genus. Thus, drugs screened against *S. mansoni* are not necessarily effective against *Schistosoma japonicum* (Vogel and Minning, 1948; Pesigan, 1951). In the present paper we have compared the activity of several drugs against *Schistosomatium douthitti*, *S. mansoni* and *S. japonicum*.

MATERIALS AND METHODS

S. douthitti, an indigenous mouse species, was originally obtained from infected snails collected near Ann Arbor, Michigan. It has been maintained in our laboratory since 1950 in laboratory-reared *Peromyscus* i.e., deer mice, and in the snails *Lymnaea stagnalis* and *Lymnaea palustris*. In our experimental work albino mice were infected by placing 50 to 60 cercariae of both sexes or 100 male cercariae on the clipped moist abdomen with a small wire loop. This method yielded uniform infections of approximately 20 to 30 worms of both sexes or 60 male worms. In over 1000 autopsies of mice exposed with this technique, one mouse was found to be uninfected (Kagan, 1952).

S. mansoni, of Puerto Rican origin, was maintained in albino mice and in the snail *Australorbis glabratus*. Each mouse was exposed percutaneously by immersion of the tail in a test tube of water containing 100 to 125 cercariae of both sexes (Oliver and Stirewalt, 1952). Approximately 15 to 25 worms per mouse were recovered.

Through the cooperation of Dr. E. B. Cram and Mr. W. B. DeWitt, Laboratory of Tropical Diseases, National Institutes of Health, we were sent 50 mice infected with *S. japonicum* of Formosan origin. These mice had been exposed in two groups to 30 to 70 or 50 to 200 cercariae placed on the clipped abdomen of each mouse and yielded 17 to 21 worms per mouse.

We tested Miracil D and three antimony compounds,* tri-(n-dodecylmarcapto)-s-antimonious acid, 2-hydroxy-4-methylol-4,5-dihydro-1,3,2-dithiastibiole and butyl antimonyl gallate. These three drugs will be referred to more briefly in the text as dodecylmarcaptide, antimony dithiastibiole and antimony gallate, respectively. Miracil D was dissolved in water, while the gallate and dithiastibiole compounds, because of their insolubility, were suspended, after being passed through a screen 100 meshes to an inch, in a 2% solution of sodium carboxymethylcellulose. All three drugs were administered orally, via stomach tube (a small piece of hollow rubber wire insulation) and syringe. The oil soluble dodecylmercaptide compound was weighed out every two days, dissolved in peanut oil and injected intraperitoneally. The amount of solvent for a single dose was 0.25 ml, except for the antimony gallate, which was suspended in 0.5 ml. Miracil D was administered in 1 or 6 doses after the methods of Kikuth and Gönnert (1948 and 1949). The antimonials were administered in 12 daily doses at slightly higher drug doses than recommended

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* The first two antimony compounds were generously supplied by the Abbott Research Laboratories and the third one by the Squibb Institute for Medical Research.

by Schubert (1948, 1949, 1950). Dosages of drugs were calculated on the basis of the average weight of the mice prior to treatment. Mice were weighed before infection, before treatment, after treatment and before necropsy. Sodium carboxymethylcellulose fed at 0.25 ml levels for 12 days to 3 mice infected with *S. douthitti* and 3 normal mice showed no therapeutic or toxic properties.

Treated mice were generally necropsied approximately two weeks after the final dose. At necropsy the blood vessels to and from the liver were ligated. The liver was removed from the body and perfused separately in a petri dish. The intestine and mesenteries were perfused through the aorta and carefully examined for worms that may not have been removed. The spleen, liver and lungs were crushed between two pieces of plate glass, 4 by 6 inches, and examined under a binocular dissecting microscope for living or dead worms, worm pigments and eggs. Worms were fixed in cold Bouin's fluid and tissues were fixed in Zenker-10% formal. Drug activity was measured by the number of living worms recovered in treated animals as compared to untreated controls. In addition, the location of worms in the hepatic portal system and the number of mice cleared of living worms were determined. We searched for dead worms, eggs and worm pigment in the liver of all cured mice to verify the presence of an original infection. In addition, eggs in the liver were studied microscopically to detect whether the miracidium was living or dead. They were considered dead in the absence of any beating of the flame cells or movement within the shell.

EXPERIMENTAL RESULTS

Experiment 1 (series 1–12), table 1.— Three lots of the antimony dithiastibiole compound were given at the rate of 1.6 to 3.0 g/kg/day. The first lot was tested against six series of mice infected with *S. douthitti* and the second and third ones against one series of mice, each series being infected with one of the three species of schistosomes. Each series consisted of 5 to 20 mice infected from 31 to 120 days.

As compared to control mice, lot A of the drug reduced the number of living worms in infections with *S. douthitti* from 90 to 100%, lot B was ineffective against douthitti, japonicum and mansoni infections, and lot C reduced the three species 79, 43 and 74%, respectively (table 1).

Of 146 mice treated, 38% died before necropsy. More mice died following treatment with lots A & B (42% and 37%) than with lot C (27%). Mortality increased to 50% when the daily drug

TABLE 1.—*Protocol of experiments 1 with 2-hydroxy-4-methylol 4,5 dihydro-1,3,2 dithiastibiole suspended in 0.25 ml sodium carboxy-methylcellulose fed in 12 oral daily doses by stomach tube and syringe.*

Lot of drug	A						B			C		
Series*	1	2	3	4	5	6	7	8	9	10	11	12
Species of schistome†	D	D	D	D	D	D	D	J	M	D	J	M
Number of mice treated	6	11	15	14	13	15	20	14	12	14	7	5
Age of infection prior to treatment in days	67	31	42	38	34	44	31	57	120	32	50	103
Average weight before treatment	26.8	25.[illegible]	23.0	24.3	23.0	23.5	25.0	21.6	22.1	24.0	23.1	30.0
Average weight before necropsy	24.0	23.3	23.0	22.0	26.5	27.0	25.0		27.0		27.4	29.0
Dosage in g/kg/day	1.6	2.1	1.9	1.7	1.8	2.0	1.8	2.0	2.0	2.0	3.0	1.7
Number of mice necropsied	4	5	9	8	13	4	17	5	7	9	5	5
Percent mice cleared of living worms	50	60	90	100	100	50	0	0	0	30	0	40
Days between end of treatment and necropsy	15	39	30	16	37	27	0–18	2–20	14–21	15	28	23
Number of control mice	15	4	7	6	4	4	8	4	1	2	5	4
Average number of worms in control mice	61.0	31.0	35.7	30.7	27.1	27.1	21.7	21.0	20.0	14.0	17.2	15.2
Average number of worms in treated mice	4.7	1.2	0.4	0	0	4.7	23.3	17.2	31.8	3.0	9.8	4.0
Percent worm reduction in treated mice	92.4	96.2	99.6	100	100	89.5	0	17.6	0	78.6	42.9	73.7

* In series 1 mice were exposed to male cercariae only; in all other series they were exposed to carcariae of both sexes. Only 11 doses were given in series 2 because of high mortality during treatment, all other series received regular 12 doses.
† D = *S. douthitti*, J = *S. japonicum*, M = *S. mansoni*.

dose was raised to 1.9 to 2.1 g/kg/day.

In addition to the foregoing series, two series of three mice each were given 9.7 g/kg in one or at two doses. When the mice were necropsied two or three days later (when four mice died), worms were found localized in the liver. As early as the second day in treatment, with much smaller drug doses, a shift of location from the mesenteries to the one mouse of series 18. Living and dead worms actually found at necropsy were largely localized in the liver, but in series 13 they were also found in the mesenteric venules and in one mouse in the spleen. Worms were also found in the lungs in 3 of 8 mice of series 17. It is of interest that the drug was ineffective against the male worms of *S. douthitti* in series 13 and of *S. japonicum* in one

TABLE 2.—*Protocol of experiment 2 with tri-(n-dodecylmercapto)-s-antimonious acid in 0.25 ml peanut oil injected intraperitoneally for 12 days.*

Species of schistosome	*douthitti*			*mansoni*	*japonicum*	
Series*	13	14	15	16	17	18
Number of mice treated	10	8	15	9	9	7
Age of infection prior to treatment in days	52	30	54	54	106	50
Average weight before treatment	28.1	27.2	30.0	25.9	25.0	23.0
Average weight before necropsy	30.8	25.0	28.5	28.0	27.1	22.1
Dosage in g/kg/day	0.07	0.09	0.13	0.09	0.16	0.20
Number of mice necropsied	10	7	12	6	8	6
Percent of mice cleared of living worms	0	87	100	83	61	50
Days between end of treatment and necropsy	14–44	17	5–37	17	6–27	0–6
Number of control mice	15	3	2	42	4	5
Average number of worms in control mice	61.0	29.3	15.5	15.2	21.0	17.2
Average number of worms in treated mice	62.6	0.4	0	0.5	1.0	2.0
Percent of worm reduction in treated mice	0	98.6	100	96.6	95.3	88.4

* In series 13 mice were exposed to male cercariae only; in all other series they were exposed to cercariae of both sexes. Mice in series 17 were used in series 8. Since these animals were passing viable eggs 38 days after the end of the first experiment they were retreated in this series.

liver was noted in mice infected with *S. douthitti* and *S. mansoni*. In infections with *S. japonicum*, however, although there was a decrease in the number of worms recovered in treated mice, in a number of mice living worms were found in the mesenteries. Nine worms in series 5 and 6 were recovered from the spleens of six mice infected with *S. douthitti*.

Experiment 2 (*series 13–18*), *table 2.*—The oil-soluble dodecylmercaptide effectively cleared mice of their worms in all series except 13, in which the dose level was slightly lower (0.07 as compared to 0.09 to 0.2 g/kg/day). In general, mice were infected with both sexes of cercariae, but only male cercariae were used in series 13 and in mouse of series 18. The drug was evidently active from the start of treatment in view of the appearance of dead worms recovered immediately after the end of treatment.

Of 58 mice treated, 15.5% died before necropsy. Practically all mice contained from 0.5 to 1.5 ml of oily fluid in their peritoneal cavity. The peritoneum was thickened and nodular in appearance, and adhesions were found between the diaphragm and liver and spleen.

Experiment 3 (*series 19–21*) *table 3.*—Butyl antimonyl gallate given at the rate of 1.4 to 2.0 9/kg/day effectively surpressed the worm burden in mice infected with *S. douthitti*, *S. mansoni* or *S. japonicum*. In series 20 (*S. mansoni*) worms were found in the liver. Living

worms in series 19 and 21 (*S. douthitti* and *S. japonicum*) were recovered from the mesenteric venules as well as from the liver. They were, however, atypical and morphologically altered.

Experiment 4 (series 22-26) table 4.—Miracil D administered at levels of 0.12 to 0.98 g/kg/day did not significantly reduce the number of living worms in 4 series tested with *S. douthitti*, but was effective at a dose level of 0.16 g/kg/day against *S. mansoni* in series 26. Of the 4 mice in series 26 necropsied 16 days after the end of treatment, 2 living worms, one each in 2 mice, were recovered from the liver,—morphologically altered and atypical in appearance. In series 22 to 25 douthitti worms were recovered chiefly from the mesenteries and, in one instance, 19 worms were recovered from the spleen. Of 41 mice treated, 63% were autopsied.

Experiment 5.—Eggs in the liver of cured mice were examined. Eggs or dead worms were found in the tissues of all mice cleared of their infections. Microscopical examinations were made of pieces of liver from 34 mice cleared of douthitti infections by antimony therapy, to determine whether the miracidia within the eggs in the tissue were alive or dead. Living eggs were observed in 9 mice autopsied 11 to 20 days after treatment began. A few living and many dead eggs were noted in 3 mice autopsied at 26, 27 and 28 days, and only dead eggs were observed in 22 mice autopsied at 24 to 37 days after treatment began. Thus, eggs of *S. douthitti* in infected, treated mice appear to have a life span of approximately 3 to 4 weeks.

TABLE 3.—*Protocol of experiment 3 with butyl antimonial gallate suspended in 0.5 ml sodium carboxymethylcellulose administered orally in 12 daily doses by stomach tube and syringe.*

Series	19	20	21
Species of schistosome	*douthitti*	*mansoni*	*japonicum*
Number of mice treated	8	6	7
Age of infection prior to treatment in days	74	87	50
Average weight before treatment	27.9	29.0	21.1
Average weight before necropsy	25.4	29.5	27.7
Dosage in g/kg/day	1.4	1.4	2.0
Number of mice necropsied	5	4	3
Percent of mice cleared of living worm	60	25	33
Days between end of treatment and necropsy	18–21	18	7–16
Number of mice for control	2	4	5
Average number of worms in control mice	15.5	15.2	17.2
Average number of worms in treated mice	1.6	2.4	1.7
Percent worm reduction in treated mice	96.1	84.2	90.1

DISCUSSION

The three antimonials studied in our

TABLE 4.—*Protocol of experiment 4 with Miracil D administered orally with stomach tube and syringe.*

Species of schistosome	*douthitti*						*mansoni*
Series*	22	23	24	25	b	c	26
Number of mice treated	5	8	15	2	2	2	7
Age of infection prior to treatment in days	77	34	35	78	78	78	66
Average weight in g before treatment	28.8	28.0	25.4	29.0	29.5	28.5	21.2
Average weight before necropsy	30.2	22.5	22.7	31.0	29.0	31.0	20.0
Dosage in g/kg/day	0.12	0.16	0.16	0.32	0.61	0.98	0.16
Number of doses	6	6	6	1	1	1	6
Days between end of treatment and necropsy	14–42	16	16	17–31	24–37	17–39	16
Number of mice necropsied	5	2	9	2	2	2	4
Percent mice cleared of living worm	0	0	0	0	0	0	50
Number of control mice	15	3	2	15	15	15	3
Average number of worms in control mice	16.0	29.3	22.3	61	61	61	25.6
Average number of worm in treated mice	54.4	27.5	20.0	47.0	50.2	68.0	0.5
Percent worm reduction in treated mice	11.8	6.2	10.4	23.0	17.7	0	98.5

* In series 22 and 25 mice were exposed to male cercariae only; in all other series they were exposed to cercariae of both sexes.

experiments were reported by Schubert (1948, 1949, 1950) to be 100% curative for mice infected with *S. mansoni* when fed in the diet. Our results with the same compounds administered orally with mansoni infections in mice were not as absolute, even though we used slightly higher dose levels. Differences in method of treatment are known to cause variation in the activity of the same compound. The size of a curative dose may depend upon the severity of infection, the extent of tissue damage, the number of worms in the host and the species of parasite. Our mice were not all cleared of worms probably because they were more heavily infected and were administered the drug differently.

Mice infected with *S. douthitti* responded well to treatment with the three antimonials. Differences between the compounds were noted and between different preparations of the same compound as shown in experiment 1. One complete failure with one of these antimonial compounds was found in series 13. We believe this may in part be associated with the lower dosage used and the presence of only male worms.

The life span of three to four weeks for douthitti eggs in treated mice suggests that eggs are not killed by treatment. Vogel (1942) observed living eggs in mice infected with *S. japonicum* 29 days after the beginning of treatment with tartar emetic, and in the tissues of untreated dogs he concluded that eggs live approximately three weeks. Bang and Hairston (1946) also demonstrated that antimonials do not affect japonicum eggs during therapy.

Infections with *S. japonicum* are more resistant to therapy than other species of schistosomes either experimentally (Vogel and Minning, 1948) or clinically (Most et al, 1950). To date there is no satisfactory treatment for this disease. Bang and Hairston (1946) reported that among common laboratory animals infections with *S. japonicum* in mice showed little drug response. Our work indicates that mice may serve as an adequate test animal for therapeutic trials. For example, in experiments 2 and 3 (series 17, 18 and 21) infected mice showed a decrease in worms of approximately 90%, and over half of them were completely cleared of their adult worms. Of the three antimonials tested, the dithiastibiole compound, when fed at almost two times the level used with *S. douthitti* or *S. mansoni*, failed to clear any mice of their adult worms but reduced the number of worms 43%. The other two antimonials were also active against experimental infections of *S. japonicum* when fed at higher dose levels than were used against the other species.

The oil-soluble dodecylmercaptide, though it produced a slight peritonitis, appeared to be the least toxic of the three antimonials. The mortality (15.5%) in mice treated with this drug fell within the range encountered in untreated mice infected for 5 to 10 weeks. The local irritation caused by the treatment may perhaps be avoided when administered by another route. The changes in body weight in mice before and after treatment were irregular and neither served as an indicator of toxicity nor therapeutic response.

Bang and Hairston (1946) reported a migration of worms from the venules of the mesenteries towards the liver after the administration of effective antimonials in dogs and guinea pigs infected with *S. japonicum*. Schubert (1948) noted a similar response in mice infected with *S. mansoni*. Our work corroborates these findings on *S. mansoni* and *S. japonicum* and extends them to *S. douthitti*. In addition, we observed worms in the lungs of treated mice in-

fected with *S. japonicum* and in the spleen of treated mice infected with *S. douthitti*.

Miracil D has been reported to be effective in the treatment of experimental mansoni infections in mice by Kikuth and Gönnert (1948 and 1949), Schubert (1948), Watson, Azim and Halawani (1948) and Halawani and Dawood (1950). A brief review of the many pharmacological and clinical reports with this drug may be found in Watson, Pringle and Jamil (1951). We found that the drug reduced the number of mansoni worms in mice by 98.5% but was ineffective against *S. douthitti*. We do not believe that the inactivity of the drug against *S. douthitti* is due to the age of the worm (34 to 35 days) prior to treatment or the time of autopsy (16 days) in series 23 and 24. Kikuth and Gönnert (1948 and 1949) reported that Miracil D was active only against mature *S. mansoni*. Since *S. mansoni* reaches sexual maturity in 35 to 40 days (Faust et al, 1934) and *S. japonicum* in 25 days (Vogel, 1942), mice would have to be infected 6 to 8 weeks to obtain mature infections. On the other hand, *S. douthitti* reaches sexual maturity in 12 days (El-Gindy, 1951), and worms are almost full grown and eggs may be recovered from the feces in 30 to 35 days. Mice were autopsied in series 23 and 24 two weeks after the end of treatment because dead worms were found 16 days after the end of treatment in mice infected with *S. mansoni*.

Miracil D has also been reported to be ineffective in the treatment of experimental japonicum infections in rabbits, hamsters and monkeys (Vogel and Minning, 1948) and in man by Pesigan (1951). Besides a comparable response of *S. douthitti* and *S. japonicum* towards antimonial compounds and Miracil D, other similarities between these two species may be mentioned, such as: both are natural parasites of rodents; eggs are similar in size and shape; cercariae are shed at night, are negatively geotropic and cling to the surface film of the water after release from the snail; approximately 40 to 60% of cercariae develop into adult worms in mice; both species produce large numbers of eggs forming egg tubercles not only in the liver but also in the spleen; and both show similar pathology of the lungs of mice (Oliver, 1952) early in infection. *S. douthitti* may serve as an extremely useful screening species for schistosomiasis japonica because it produces no systemic infection in man, is found in many parts of the United States and is relatively easy to maintain in the laboratory on a large scale.

We were interested in obtaining complete cures and treated animals in some instances with drugs above the tolerated dose. The drug that showed the most promise in this regard was the oil-soluble antimonial. This compound warrants further study. Butyl antimonial gallate also significantly decreased the number of living adult worms and those that remained were abnormal. This drug might yield interesting results if fed at higher levels or if the mice were necropsied later. The dithiastibiole compound in a majority of tests was found to be active in decreasing the number of adult worms in mice. Miracil D was not active against *S. douthitti* but was very effective against *S. mansoni*.

SUMMARY

Three antimony compounds, 2-hydroxy-4-methylol-4,5, dihydro-1,3,2, dithiastibiole, butyl antimonyl gallate (fed orally) and tri-(n-dodecylmercapto)-s-antimonious acid (injected intraperitoneally in peanut oil), and Miracil D

(fed orally) were tested against infections in mice with *Schistosomatium douthitti*, *Schistosoma mansoni* and *Schistosoma japonicum*.

Our work indicates that the antimonial drugs reduced infection with *S. mansoni* 74 to 99%, and at slightly higher dose levels reduced infection with *S. japonicum* 43 to 95%, and that infections with *S. douthitti* were reduced 79 to 100%. Miracil D proved to be effective against *S. mansoni* and ineffective against *S. douthitti*.

The oil-soluble antimonial, since it was the most effective against all three species, warrants further study. It evidently begins to act early, as indicated in the appearance of dead worms from mice autopsied after treatment had been stopped.

Aside from killing adult worms, the drugs caused worms to move from the mesenteric venules towards the liver. They appeared not to harm the eggs of *S. douthitti* directly.

Morphological and biological similarities between *S. douthitti* and *S. japonicum* are discussed. It is suggested that *S. douthitti* may be a useful screening model for schistosomiasis japonica.

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THE RECOVERY OF STRAINS OF ROCKY MOUNTAIN SPOTTED FEVER AND TULAREMIA FROM TICKS OF THE EASTERN UNITED STATES

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A survey of parasites and diseases of small mammals and their blood-sucking parasites in certain sections of the eastern United States was carried on during the last half of 1939 and the first half of 1940 by the School of Animal Pathology of the University of Pennsylvania.[1] The ticks collected were tested at the Rocky Mountain Laboratory for the presence of *Bacterium tularense* and rickettsial disease agents (see table 1).

Each group consisted of ticks of one species and, with few exceptions, was from one host. The smallest group contained 2 ticks, the largest, 158, and the average, 32.

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All the *Haemaphysalis leporis-palustris* (rabbit ticks), the *Ixodes dentatus*, and the one group of *Amblyomma americanum* were collected from cottontail rabbits. Of the 113 cottontails examined 51 were infested with both *H. leporis-palustris* and *I. dentatus*, 35 with only the former, and 27 with only the latter. The largest number of *H. leporis-palustris* from one host was 158; the largest number of *I. dentatus* was 225. The majority of the *Dermacentor variabilis* were from dogs, but one group was from a cottontail rabbit, one from man, one from sheep, and two from vegetation.

Six proved strains of Rocky Mountain spotted fever were obtained from *H. leporis-palustris* and one from *I. dentatus*. In addition, three groups gave results suggestive of low-grade strains of Rocky Mountain spotted fever. Three strains of tularemia were recovered from *H. leporis-palustris* and one from *I. dentatus* (see fig. 1).

METHODS

The technique used in testing the ticks for strains of rickettsial and bacterial agents was that routinely employed for this purpose at the Rocky Mountain Laboratory. The ticks were

TABLE 1.—*Ticks tested at Rocky Mountain Laboratory.*

Species	Groups	Number of ticks
H. leporis-palustris	91	3368
I. dentatus	83	2201
D. variabilis	16	536
A. americanum	1	9
Totals	191	6114

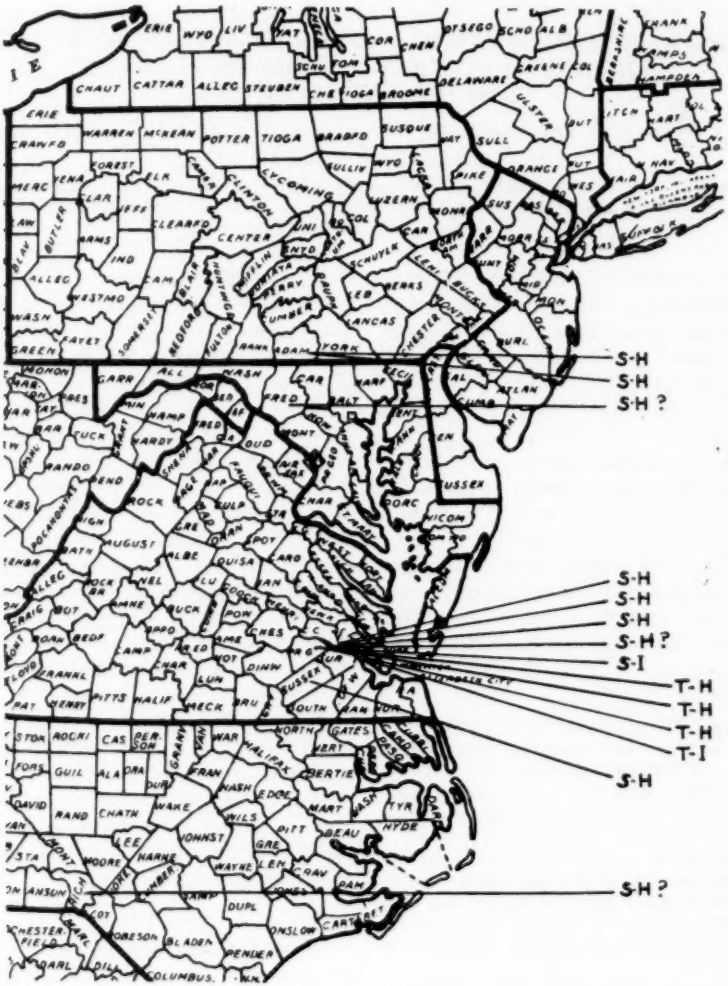

FIG. 1.—Areas from which infected ticks were obtained.
S = spotted fever, T = tularemia, S? = low-grade strain of spotted fever, H = *H. leporis-palustris*, I = *I. dentatus*.

first immersed in 1:1000 merthiolate solution for 2 to 4 hours to rid the exteriors of microorganisms. They were then rinsed in several changes of sterile physiological saline to remove all traces of the merthiolate. Next they were triturated with saline in a sterile mortar with the aid of sterile alundum. In testing, the resultant suspension was injected into 2 male guinea pigs, one pig being injected subcutaneously, the other intraperitoneally.

When a tularemia infection was suspected, heart blood was taken for culture from one of the test guinea pigs while moribund. The animal was then sacrificed, and two fresh guinea pigs were inoculated by the usual spleen-to-abraded-skin transfer. A passage strain was maintained in this

manner until a pure culture of *Bact. tularense* was obtained and was shown to be agglutinated to titer by a known antiserum.

However, there were 187 groups of ticks from which *Bact. tularense* was not recovered. The 187 pairs of guinea pigs that received these tick-tissue suspensions reacted in three ways: (a) one guinea pig of the pair was febrile (a temperature above 39.6 C), the other afebrile; (b) both were febrile; or (c) both were afebrile. If only one was febrile it was usually sacrificed, usually on the 1st, 2nd or 3rd day of fever, and spleen tissue was transferred to 2, 4, or more guinea pigs, half being injected subcutaneously, half intraperitoneally. Before the donor animal was sacrificed, heart blood was drawn and tested in 2 tubes of nutrient broth. If growth appeared in the broth, and the course of infection and the gross lesions in the transfer guinea pigs suggested definitely that other than a known or possible tick-transmitted infection was present, the test was discontinued. If, on the other hand, the tubes of broth failed to show growth, and the course of infection and the nature of the lesions in the transfer animals were suggestive of a tick-transmitted disease, a spleen-tissue passage strain was maintained, if possible, until sufficient recovered animals were on hand to permit an adequate number and variety of immunity tests, and other tests as necessary, to determine the nature of the infectious agent. Immunity tests were made for Rocky Mountain spotted fever with an intraperitoneal challenge inoculum of 1 ml of citrated heart blood taken on the 3rd day of fever from a guinea pig infected with a highly virulent *Dermacentor andersoni* passage strain. If either guinea pig remained afebrile, it received, 12 to 20 days later, a second injection of the same disease agent. Each lot of rickettsiae used for immunity tests was controlled in 2 fresh guinea pigs.

Every third passage, the heart blood of the donor guinea pig was cultured for bacteria; so also was the heart blood of each guinea pig used as the source of tissue (blood or spleen) used in immunity tests.

If both guinea pigs injected with tick tissue became febrile, only one was sacrificed in most instances, the subsequent procedure being exactly as that just outlined. If the second was also sacrificed, as happened several times, the procedure was again the same.

In the event that both tick-tissue injected guinea pigs remained afebrile, each received immunity tests in the manner which has been described for passage guinea pigs. So also did surviving mates of animals that were sacrificed to initiate passage strains.

Since virulent strains of Rocky Mountain spotted fever have not been previously recorded from *H. leporis-palustris*, the data relative to virulence of the strains obtained in these studies are presented in detail.

RESULTS

The specificity of the 4 strains of *Bact. tularense* that were recovered (13-6, 13-14 and 13-14a from *H. leporis-palustris* and 2-8 from *I. dentatus*) was established by the means already described.

Five of the 7 proved strains of Rocky Mountain spotted fever recovered from *H. leporis-palustris* and *I. dentatus* were experimentally observed through 15 passages. One or more substrains of each of these 5 strains were maintained for several passages, using blood as the transfer medium. Several attempts were made to initiate substrains by the transfer of testicular washings. Only 1 of 3 strains of Rocky Mountain spotted fever (2–27) tested could be passed, and that for only one passage.

Two of the Rocky Mountain spotted fever strains from *Haemaphysalis* were lost, one (13-7) after the first passage, the other (13-21) after the sixth.

Nine different highly virulent *D. andersoni* strains of Rocky Mountain spotted fever were used for the numerous Rocky Mountain spotted fever immunity tests. All of the guinea pigs which were inoculated with the various strains of spotted fever and recovered were immune when subsequently challenged with the virulent stock strains.

Four of the rickettsial strains that immunized against Rocky Mountain spotted fever (three *H. leporis-palustris*, 13-30, 13-37, and 13-74, and one *I. dentatus*, 2-27) were also tested against Rocky Mountain spotted fever vaccine. Twenty-four guinea pigs each received 1 ml of vaccine 8313. Ten days later each of a group of six of these animals and three fresh controls received one

of these rickettsial strains (1 ml of spleen tissue suspension intraperitoneally). In each instance all six test animals remained completely afebrile while all control animals had typical febrile reactions.

Another of the *Haemaphysalis* strains that immunized against Rocky Mountain spotted fever (13-35) was tested against several rickettsial agents. Twenty-five guinea pigs received this strain and all were typically febrile and recovered. Twenty-nine days later five received the rickettsiae of Minas Gerães typhus (295th guinea pig passage),* five that of the original strain of American Q fever (213th passage), five that of endemic typhus (Wilmington strain, 352d passage), five that of *I. dentatus* strain 2-27 (15th passage), and five that of "*maculatum*" infection (strain 6-1, third passage).[2] Each challenge inoculum was controlled in three guinea pigs. None of the guinea pigs that received challenges with American Q fever and endemic typhus were immune. All five were immune to Minas Gerães typhus (Brazilian spotted fever), to the *I. dentatus* strain, and three of five to "*maculatum*" infection (the tests in two animals were valueless).

Both mortality and the incidence of scrotal lesions were very low among the guinea pigs that were injected with the several strains of Rocky Mountain spotted fever. There were no deaths and no scrotal lesions among the guinea pigs used for strains 13-7 and 13-21, which were lost early. Two hundred and sixty-seven transfer guinea pigs were inoculated in maintaining strains 13-30, 13-35, 13-37, 13-74, and 2-27 for 15 passages. One hundred and fifty-three of these guinea pigs (others were sacrificed for transfer material or other studies) were carefully observed for scrotal signs. There were 12 that developed some degree of swelling and reddening of the scrotum. In most of this group the reaction was ill-defined and transitory, but in one guinea pig there was sloughing and in another the reac-

TABLE 2.—*Strains of Rocky Mountain spotted fever and tularemia recovered from ticks collected in the eastern United States and three low-grade strains of Rocky Mountain spotted fever.*

Test and strain no.	Tick species	Date collected	State	County	Test data	
					Number of ticks tested*	Infection recovered
13-7	*H. leporis-palustris*	3-29-40	Virginia	Prince George	17	Rocky Mountain spotted fever
13-21	*H. leporis-palustris*	4-16-40	Virginia	Prince George	62	Rocky Mountain spotted fever
13-30	*H. leporis-palustris*	4-25-40	Pennsylvania	Adams	63	Rocky Mountain spotted fever
13-35	*H. leporis-palustris*	4-26-40	Pennsylvania	Adams	19	Rocky Mountain spotted fever
13-37	*H. leporis-palustris*	4-24-40	Virginia	Prince George	80	Rocky Mountain spotted fever
13-74	*H. leporis-palustris*	5-20-40	Virginia	Sussex	56	Rocky Mountain spotted fever
13-6	*H. leporis-palustris*	3-28-40	Virginia	Prince George	68	Tularemia
13-14†	*H. leporis-palustris*	4- 9-40	Virginia	Prince George	50	Tularemia
13-14a†	*H. leporis-palustris*	4- 9-40	Virginia	Prince George	50	Tularemia
13-9	*H. leporis-palustris*	4- 8-40	Virginia	Prince George	58	Low-grade strain of Rocky Mountain spotted fever
13-12	*H. leporis-palustris*	4- 2-40	North Carolina	Richmond	32	Low-grade strain of Rocky Mountain spotted fever
13-58	*H. leporis-palustris*	5- 2-40	Virginia	Frederick	18	Low-grade strain of Rocky Mountain spotted fever
2-8‡	*I. dentatus*	4- 9-40	Virginia	Prince George	18	Tularemia
2-27	*I. dentatus*	4-24-40	Virginia	Prince George	7	Rocky Mountain spotted fever

* Each group of ticks tested was from one cottontail rabbit.
† A strain of tularemia was recovered from *I. dentatus* from same rabbit.
‡ Two strains of tularemia recovered from *H. leporis-palustris* from same rabbit.

* There is complete cross immunity between this Brazilian disease and Rocky Mountain spotted fever.

2. Parker, R. R., Kohls, G. M., Cox, G. W. and Davis, G. E. 1939, Observations on an infectious agent from *Amblyomma maculatum*. Pub. Health Rep. **54**: 1482–1484.

tion was marked. Among the same 267 transfer guinea pigs there were five deaths that may have been due solely to Rocky Mountain spotted fever. However, it is of interest to note that the mates of the guinea pigs from which passage strains 13-30 and 2-27 were initiated both died of apparent spotted fever infection, whereas no deaths occurred among 63 passage guinea pigs of the former strain and only 1 among 71 of the latter strain.

Dr. Herald R. Cox, at the Rocky Mountain Laboratory, initiated chick embryo passage strains of Rocky Mountain spotted fever strains 13-35 and 2-27 from the 8th and 10th passage guinea pigs, respectively. The staining properties, morphology and general appearance of the rickettsiae, as observed by him in smears of yolk-sac tissue, were identical with those of our stock strains, as were also the reactions in guinea pigs inoculated with chick-embryo tissues.

It has previously been stated that tests of three groups of *H. leporis-palustris* gave results suggestive of the possible presence of low-grade strains of Rocky Mountain spotted fever (table 2). Two of the three test guinea pigs concerned (tests 13-12 and 13-58) remained completely afebrile following the injection of the tick-tissue suspension; one of them (test 13-9) was febrile only on the 7th day (40.0 C). Two each received 2 subsequent immunity tests of Rocky Mountain spotted fever rickettsiae; the 3rd received but one. One of those (13-9) that received 2 tests had one day of fever following each test. These was no fever following the tests given the other 2. Each lot of rickettsiae used for these immunity tests was also used to test the immunity of 20 or more other guinea pigs (unrelated experiments) and in 2 controls. Typical severe spotted fever developed in all of them. For example, lot 1349 was used to test 51 other guinea pigs (including some not of this series of tests) that had not previously received material containing Rocky Mountain spotted fever rickettsiae. Each one had a typical febrile reaction (41 died), and all male animals also showed typical scrotal involvement.

Temperature records of tick-tissue injected guinea pigs in which proved strains of Rocky Mountain spotted fever were recovered from *H. leporis-palustris* and *I. dentatus* are given in table 3. These guinea pigs were sacrificed on the last day for which the tem-

TABLE 3.—*Temperature records of tick-tissue infected guinea pigs in which proved strains of Rocky Mountain spotted fever were recovered from* H. leporis-palustris *and* I. dentatus.

Day after injection	*H. leporis-palustris* strains						*I. dentatus*
	13–7	13–21	13–30	13–35	13–37	13–74	2–27
1	39.6	39.6	39.0	39.2	38.8	38.8	38.8
2	40.0	39.6	39.2	39.4	39.0	38.6	39.0
3	38.8	39.6	39.0	39.0	39.0	39.0	39.0
4	39.0	38.8	38.8	38.6	39.0	38.8	39.0
5	39.0	39.2	40.6	39.0	39.2	39.0	40.2
6	39.2	39.6	40.6	39.4	40.2	40.4	41.0 S
7	39.0	39.0	41.0 S	40.2	41.0 S	40.2	Spl. ×4.0
8	39.0	39.0	Spl. ×4.0	40.4 S	Spl. ×3.0	40.4 S	
9	38.8	39.2		Spl. ×3.0		Spl. ×2.0	
10	39.0	39.6					
11	40.2	40.8					
12	40.0	40.6					
13	40.4 S	40.6 S					
14	Spl. ×1.5	Spl. ×3.5					

S = sacrificed.
Spl. ×1.0 = spleen enlarged 1.0 times.

perature is recorded, and transfer was made to fresh guinea pigs by spleen-tissue suspension. Heart blood taken from each animal before sacrifice was sterile.

DISCUSSION

The series of tests described in the foregoing pages resulted in the following data: (1) isolation of a strain of Rocky Mountain spotted fever from *I. dentatus*, (2) isolation of a strain of tularemia from *I. dentatus*, and (3) isolation from *H. leporis-palustris* of strains of Rocky Mountain spotted fever that produce frank infections that can be maintained in guinea pigs.

The apparent involvement of *H. leporis-palustris* as an agent in the natural maintenance of *Bact. tularense* and of the rickettsia of Rocky Mountain spotted fever in the western states and western Canada has been known for some years.[3] With respect to *Bact. tularense* it has been quite naturally assumed that *H. leporis-palustris* functions in the same capacity in the east. Our data afford the first proof that this is definitely true. In the case of Rocky Mountain spotted fever, however, such an assumption has had less justification, and it is felt that the evidence that this association does actually occur in the east has some potential interest.

The specificity of the six strains of Rocky Mountain spotted fever recovered from *H. leporis-palustris* and the one from the *I. dentatus*, all of which provoked frank infection, was established by showing that they immunize guinea pigs against highly virulent *D. andersoni* strains, that Rocky Mountain spotted fever tick-tissue vaccine fully protects guinea pigs against them, and that, when grown in the embryonic tissues of developing chicks, the rickettsiae exhibit all the characteristics of those of known Rocky Mountain spotted fever strains cultured similarly. The tests against Rocky Mountain spotted fever vaccine were essential to eliminate the rather unlikely possibilities that we are dealing with strains of boutonneuse fever* or of "*maculatum*" infection.[2] The former always immunizes against Rocky Mountain spotted fever strains of the highest virulence; the latter is slightly less consistent, but spotted fever vaccine does not protect against either.

In addition, *Haemaphysalis* strain 13-35 was shown to immunize against Minas Gerães exanthematic typhus, a Brazilian disease that is immunologically identical with Rocky Mountain spotted fever, and against "*maculatum*" infection.

Four of the six proved *H. leporis-palustris* strains of Rocky Mountain spotted fever differ from any of the strains of the infection previously met with in this species of tick (see next paragraph) in that they produce frank infections and can be maintained by serial passage in guinea pigs. They differ from the highly virulent *D. andersoni* strains prevalent in western Montana in their relatively low virulence, the infrequency with which they provoke scrotal lesions, the fact that the spleen is usually less enlarged and less smooth, and that they are more readily maintained by transfer of spleen tissue than by transfer of blood and cannot be maintained by the transfer of testicular washings. In these respects, these *Haemaphysalis* strains and the *I. denta-*

3. Parker, R. R., Pickens, E. G., Lackman, D. B., Bell, E. J. and Thrailkill, F. B. 1951, Isolation and characterization of Rocky Mountain spotted fever rickettsiae from the rabbit tick *Haemaphysalis leporis-palustris* Packard. Pub. Health Rep. **66**: 455–463.

* While boutonneuse fever has not been reported in this country, its proved vector in the Mediterranean littoral, *Rhipicephalus sanguineus*, is present in the United States and occurs more or less sporadically in the states herein concerned.

tus strain agree with the usual strains obtained from *D. variabilis* as they have been observed at the Rocky Mountain Laboratory.

The question of whether or not the results of the three *Haemaphysalis* tests, 13-9, 13-16, and 13-58 (table 2), indicate that the ticks contained low-grade Rocky Mountain spotted fever rickettsiae depends on whether or not the particular 1 ml units of spotted fever blood used for the first immunity tests actually contained virulent rickettsiae. It is probably safe to assume that at least two of the three tests were valid.

It is essential to point out that our recovery of strains of Rocky Mountain spotted fever and tularemia from tick species not heretofore known to be implicated in their transmission does not mean that these species are necessarily vectors of these infections. It is reasonably probable that they are, but their unqualified acceptance as such must await experimental proof that the infectious agent of each disease can survive in the species of ticks respectively concerned and that it can be transmitted by at least one or more of the stages that follow the one by which the disease agent is ingested.

SUMMARY

One hundred and ninety-one groups of ticks from Pennsylvania, Virginia, West Virginia, New York, Connecticut, North Carolina, and Alabama were tested for the presence in their tissues of strains of *Bacterium tularense* and rickettsial disease agents. There were 91 groups and 3368 specimens of *Haemaphysalis leporis-palustris*, 83 groups and 2201 specimens of *Ixodes dentatus*, 16 groups and 536 specimens of *Dermacentor variabilis*, and 1 group of 9 specimens of *Amblyomma americanum*.

Eleven strains of infectious agents were recovered. Of four strains of tularemia, all were from Prince George County, Virginia—three from *H. leporis-palustris* and one from *I. dentatus*. Of seven strains of Rocky Mountain spotted fever, six were from *H. leporis-palustris* and one from *I. dentatus*. Two of the six *Haemaphysalis* strains were from Adams County, Pennsylvania, three from Prince George County, Virginia, and one from Sussex County, Virginia. The single *Ixodes* strain was also from Prince George County. Tests of three groups of *Haemaphysalis* suggested the possible presence of low-grade strains of Rocky Mountain spotted fever.

Strains of Rocky Mountain spotted fever that will provoke frank infections in guinea pigs and that can be maintained in them by serial passage have been isolated from *H. leporis-palustris* for the first time.

FRACTIONS OF BRUCELLA FOR ADSORBED ANTIGENS FOR COLLODION AGGLUTINATION AND HEMAGGLUTINATION TESTS

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The limitations of the standard agglutination test for the diagnosis of brucellosis are well known and have been described by many authors, among them Huddleson (1943), Griggs and Case (1948), and Steele and Emik (1950). The great variation in the performance of these tests using so-called standard methods has indicated the need for a better diagnostic test. Furthermore, the difficulty of isolating brucella from blood cultures and the rare positive findings obtained in highly suspected cases has emphasized the need for a good test.

The recent work by Middlebrook and Dubos (1948), Smith and Scott (1950) and others on the hemagglutination test for tuberculosis has stimulated the application of the hemagglutination technique to the diagnosis of other diseases. Although the possibility of extracting substances from brucella which might be used for this method was not promising (Feinberg, 1951), the fact that antigenic extracts of brucella have been produced over a long period of years by Miles and Pirie (1939a and b), Paterson et al (1947), Elberg et al (1950 and 1951), and Silverman and Elberg (1950), stimulated the effort to find fractions which could be adsorbed onto sheep cells or some other colloid and thus provide a diagnostic test of greater specificity and sensitivity than the standard bacterial agglutination test. This investigation describes the isolation of fractions of brucella using simple methods, and the adsorption of these extracts onto sheep cells and onto collodion for the hemagglutination and collodion adsorption agglutination tests.

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MATERIALS AND METHODS

Preparation of extracts

I. *Saline extracts of killed brucella.*
Larson (1951) has used saline extraction of *Pasteurella tularense* to produce antigens. Mixed strains of brucella killed with 0.5% phenol were washed once in 0.85% sodium chloride and resuspended in fresh saline. Samples for testing were withdrawn at weekly intervals for a month and at monthly intervals for 7 months; the final extract was tested after 2 years. The samples were centrifuged until clear or very slightly opalescent.

II. *Saline extracts of ground killed brucella.*
A. The organisms were ground varying lengths of time in an agate mortar or in a ball mill. The ground organisms were suspended in saline. Samples were removed at hourly intervals for 4 hours, at 24 hours and at 48 hours, at weekly intervals for 4 weeks, and at monthly intervals for 7 months. The samples were centrifuged until clear or slightly opalescent.
(1) Organisms ground in an agate mortar for 45 minutes.
(2) Organisms ground in a ball mill for 48 hours.
B. One portion of these saline extracts was treated with *N*/20 hydrochloric acid following Wadsworth's (1947) method used to prepare streptococcus extracts for precipitation tests.
C. Second portion of the saline extract was treated with *N*/2 trichloracetic acid.
(1) The very small amount of precipitate was dissolved in 1% sodium carbonate neutralized with hydrochloric acid and 0.85% sodium chloride added.
(2) The extract was dialyzed in cold running water for 5 hours, evaporated to dryness, and taken up in a small amount of saline.

D. The trichloracetic acid solution from this treatment was dialized 2 days in cold running tap water and 2 days in distilled water. Three types of material were obtained:
   (1) Boivin-type precipitate which was suspended in fresh saline.
   (2) Boivin-type precipitate shaken with glass beads for several hours, centrifuged and the suspending fluid tested.
   (3) The supernatant from the dialyzed trichloracetic acid extract made isotonic.

III. *Viable washed brucella extracted with 3* M *glycine 18 hours at 37 C.*
Cowles (1947) and Maculla and Cowles (1948) have used glycine to disrupt bacterial cells. The method is effective on viable cells only.
   A. The first saline washing from the viable brucella.
   B. After standing for 24 hours at 37 C the brucella-glycine mixture was filtered and the filtrate tested.
   C. One portion of the filtrate was dialyzed 3 days against cold running tap water and the clear, light tan-colored solution was made isotonic with sodium chloride.
   D. A portion of the filtrate was treated with *N*/2 trichloracetic acid. The precipitate was dissolved in 1% sodium carbonate, neutralized with hydrochloric acid and made isotonic with sodium chloride.
   E. A portion of the filtrate was shaken 6 times with chloroform. Three layers resulted:
      (1) A chloroform layer which was discarded.
      (2) A thick viscous layer, biuret positive, which was suspended in saline.
      (3) The glycine layer which was thrown into two volumes of cold ethyl alcohol:
         a. A precipitate resulted which appeared to be the glycine reprecipitated. This was dissolved in saline.
         b. The alcohol-glycine solution from which the alcohol was evaporated was made isotonic with sodium chloride.
   F. The sediment from the glycine treated brucella was
      (1) Suspended in saline.
      (2) Centrifuged and the clear supernatant tested.
      (3) Dialized against cold running tap water for 16 hours, centrifuged and made isotonic.
      (4) The clear supernatant poured into cold ethyl alcohol and the resulting precipitate dissolved in saline.

IV. *Broth from phenol-killed brucella cultures.*
   A. The broth was filtered through a Seitz filter (EK filter pad).
   B. Filtered broth dialyzed against cold running tap water for 24 hours and made isotonic with sodium chloride.
   C. Filtered broth mixed with two volumes cold ethyl alcohol at 4 C for 24 hours.
      (1) The precipitate dissolved in saline.
      (2) The supernatant evaporated to dryness and the residue taken up in a small amount of saline.

V. *Dried killed brucella extracted with concentrated phenol.*
Described by Palmer and Gerlough (1940), 88% phenol was used and the mixture stirred constantly with an electric mixer for 20 hours.
   A. The phenol solution was poured into two volumes of cold ethyl alcohol. The resulting precipitate was washed three times with cold alcohol, suspended in distilled water and dialyzed for 4 days against cold running tap water.
      (1) A gum-like substance precipitated. It was not soluble in 95% ethyl alcohol, ether, acetone, water or 1% sodium carbonate. It was partly soluble in *N*/2 hydrochloric acid at 56 C. The soluble portion was neutralized with sodium hydroxide.
      (2) The supernatant dialyzed phenol-alcohol solution was evaporated to dryness and redissolved in a small amount of saline.
   B. The sediment from the phenol-treated brucella was suspended in 100 cc of an aqueous solution containing 0.55% sodium chloride, 0.5% sodium phosphate and 20% methyl alcohol, stirred 20 hours with an electric mixer, centrifuged at 4000 r.p.m. for 1 hour. The mixture was adjusted to pH 6.0 and dialyzed 18 hours against cold running tap water and 2 days against distilled water. It was then evaporated to one-half volume in a cellophane bag in front of an electric fan at room temperature and made isotonic with sodium chloride.

VI. *Washed, killed brucella extracted with 20 volumes of 2% neutral phenol in the refrigerator at 4 C.*

This approximates the "native antigen" described by Miles and Pirie (1939a and b), Pennell (1950), Elsberg et al (1950 and 1951), and Silverman and Elberg (1950). An ultra centrifuge was not available and it was determined to remove the phenol by dialyzing although the possibility of losing active material was obvious. The suspensions were dialyzed overnight, 16 hours in cold running tap water, using Dupont #300-A, P. T. cellophane. Tests for phenol were positive up to 10 hours but were negative after 16 hours. The suspensions were centrifuged until clear or very slightly opalescent.

A. Ten-day extraction with phenol.
   (1) Whole organisms.
      a. The supernatant from the dialyzed phenol extraction made isotonic.
      b. The sediment suspended in saline, shaken with glass beads for several hours.

B. Thirty-day extraction.
   (1) Whole organisms.
      a. The supernatant from the dialyzed phenol extraction made isotonic.
      b. The sediment suspended in saline, shaken with glass beads for several hours.
   (2) Organisms ground in ball mill for 48 hours.
      a. The supernatant from the dialyzed phenol extraction made isotonic.
      b. The sediment suspended in saline, shaken with glass beads for several hours.

Preparation of strains

The first group of experimental antigens was made from the following strains: *Brucella* N. C. and K8, strains of *Brucella melitensis* isolated in our laboratory about 10 years ago; U. S., a strain of *Brucella abortus* received from the U.S.P.H.S. about 1935, and K, a strain of *Brucella suis* isolated in our laboratory several years ago. All of these strains with the exception of strain U. S. grew luxuriantly; U. S. remained difficult to cultivate. The mass cultures were made in bottles of trypticase soy broth, tryptose phosphate broth or trypticase soy broth with 0.5 mg percent thiamine chloride added as suggested by Huddleson (1948). Later trypticase soy agar was used; no difference could be detected between antigen prepared from organisms grown in broth and those cultivated on agar.

Other cultures were added as follows: #49, a strain of *Br. abortus* isolated this year in our laboratory, #1119, a strain of *Br. abortus* obtained from the Bureau of Animal Industry, and #89, a strain of *Br. suis* isolated this year in our laboratory.

Preparation of sheep cell suspensions

Fresh sheep cells were obtained each week in Alsever's solution. The cells were centrifuged and washed three times in 0.85% sodium chloride prepared daily from freshly distilled water and C. P. sodium chloride. A 1.0% solution was prepared by adding 1 ml of freshly washed sheep cells to 99 ml of freshly prepared saline. This suspension was used for one day only.

Preparation of collodion suspension

Collodion particles have been used for many years as a particulate base for soluble antigens. Goodner (1941), Pearson (1944), Cavelti (1944), Donaldson and Clark (1945), and Boand and Kempf (1951) have used the method to advantage. Cavelti (1947) has described a method of preparation of the collodion particles which has been followed by most investigators.

A greatly simplified method of preparing collodion particles, suggested by Angela (1950), yielded suspensions of uniform size, stable in distilled water solution and exhibiting no spontaneous agglutination in saline for several days. The stock suspension in distilled water may be used for a month.

One hundred ml of U.S.P. collodion were poured slowly into 2 liters of distilled water. The water was stirred constantly with an electric stirrer. The collodion collected on the surface of the water and could be picked up and dried out. Very little of the collodion was lost. A solution of 0.2% collodion in acetone was made.

The collodion-acetone solution was forced into a solution of 25% acetone in distilled water, using a 20-ml luer syringe with a 25-gauge needle. The acetone-water mixture was stirred constantly, using an electric stirrer. The resulting collodion suspension was placed in the incubator or in front of a fan until the acetone was evaporated off. Any specks of unsuspended collodion were skimmed off the surface.

The resulting suspension contained collodion particles ranging from 0.5 mμ to 1.5 mμ in diameter. The suspension was centrifuged for 1 minute at 4000 r.p.m., the larger particles thus sedimented and discarded. The suspension was then centrifuged for 20 minutes at 4000 r.p.m. and the sedimented particles resuspended in freshly prepared 0.85% sodium chloride.

The suspension was diluted to correspond to a #4 MacFarlane standard.

Preparation of adsorbed antigens

1. Sheep cell adsorption.—Equal parts of antigen and 1% sheep cell suspension were mixed and allowed to stand 2 hours in a 37 C water bath.

2. Collodion adsorption.—Equal parts of collodion suspension (MacFarlane standard #4) and antigen were mixed and allowed to stand 30 minutes at room temperature. Very little more adsorption took place after longer times or at 37 C. Centrifugation of the adsorbed collodion particles and resuspension in saline gave positive reactions, indicating that the antigen had been firmly adsorbed.

blocking factor described by Schuhardt et al (1951). The serums were then diluted 1:2 with saline and 0.1 ml of packed washed untreated sheep cells were added per ml of diluted serum. The serums were allowed to stand for 30 minutes at room temperature, centrifuged, the cells discarded and the procedure repeated three times or until the antisheep antibodies were completely removed.

Performance of agglutination tests

Twofold dilutions of serum were made in numbered test tubes, using freshly prepared saline. Equal amounts of the adsorbed antigens were

TABLE 1.—*Enhancement of agglutination titers by centrifugation.*

Antiserum	Antigen	Reading	Serum dilution 10	20	40	80	160	320	640	1280	Control
Immune rabbit serum (mixed)	IID(3) plus collodion	Direct	4	4	1	—	—	—	—	—	—
		Centrifuged	4	4	4	4	2	—	—	—	—
Commercial anti-melitensis serum	VIB(1a) plus collodion	Direct	3	2	—	—	—	—	—	—	—
		Centrifuged	4	4	3	2	—	—	—	—	—
Commercial anti-melitensis serum	IIIF(1) plus collodion	Direct	4	3	2	1	—	—	—	—	—
		Centrifuged	4	4	4	4	4	—	—	—	—
Commercial anti-melitensis serum	VIF(1) plus collodion	Direct	—	—	—	±	4	4	4	±	—
		Centrifuged	±	±	2	4	4	4	4	4	—
Commercial anti-melitensis serum	IID(3) plus sheep cells	Direct	—	—	—	—	—	—	—	—	—
		Centrifuged	4	4	3	3	3	—	—	—	—

Preparation of serums

1. Brucella antiserum was prepared by inoculating large rabbits with a heavy suspension of mixed dead brucella. One ml of a 5 billion per ml suspension was injected intraperitoneally. Two days later 0.1 ml was injected intravenously. Two days later and every other day thereafter for 4 injections, 0.5 ml was injected intravenously. The animals were bled 9 days after the last injection, the serum separated, tested, and stored in the deep-freeze without preservative.

2. Commercial Markham laboratory anti-*Br. abortus* and anti-*Br. melitensis* serums.

3. Human serums obtained from specimens sent to our laboratory for brucella agglutination tests and clot cultures. We are grateful to Dr. Charles Carpenter, the Gilmour Hodges Clinic, and Dr. Ralph Morgan for specimens from patients presumed to have brucellosis. The serums were stored in the freezing compartment of the refrigerator.

Serums containing merthiolate in concentration near or above 1:1000 gave somewhat variable results. It was found that collodion particles were spontaneously agglutinated by high concentration of merthiolate.

Before use, the serums were inactivated at 56 C for one-half hour. This removed the heat-labile

added, the tests shaken well and incubated in a 37 C water bath for 24 hours. The tests with collodion adsorbed antigens were shaken after 4 hours; those with sheep cell adsorbed antigens were shaken at hourly intervals for the first 5 hours of incubation.

After incubation, the collodion tests were examined for evidence of clumping, shaken thoroughly and the tubes centrifuged for 3 minutes at 1600 r.p.m. in a #1 International centrifuge. The tests were read by transmitted natural light. Complete agglutination with clear supernatant was designated a 4+, partial agglutination a 3, 2, or 1+ reaction.

The sheep cell tests were examined first for evidence of agglutination of the chicken-hemagglutination-influenza test type. Such findings were rare. The tubes were shaken thoroughly, then centrifuged as in the collodion tests and read.

Table 1 shows the difference in reading between centrifuged and noncentrifuged tests.

RESULTS

In the beginning it was felt that the antigenic fraction which might be adsorbed onto sheep cells or collodion

TABLE 2.—*Results of agglutination reactions with adsorbed antigens.*

Brucella extracts	Ring precipitin test	Agglutination reactions		Antigen nitrogen content/ml
		Collodion adsorbed antigens	Sheep cell adsorbed antigens	
I. Saline extracts killed brucella	+	−	−	0.127
II. Saline extract ground killed brucella				
A (1)*	+	−	−	
(2)	+	−	−	0.039
B	+	−	−	
C (1)	−	−	−	0.096
(2)	+	+	+	0.083
D (1)		+	Unsatisfactory	
(2)	−	+	Weakly +	0.075
(3)	−	+	+	0.028
III. Viable brucella extracted with glycocoll				
A			−	
B	−	Weakly +	−	
C	−	−	−	0.07
D	−	−	−	
E (2)	−	−	−	
(3a)	−	−	−	
(3b)	−	−	−	
F (1)	−	−	−	
(2)		Weakly +	Hemolyzed	0.109
(3)		False +	Hemolyzed	
(4)		+	Hemolyzed	0.049
IV. Broth from brucella cultures				
A	−	−	−	
B	−	−	−	0.190
C (1)	−	−	−	
(2)	−	−	−	
V. Concentrated phenol extracted brucella				
A (1)		+	−	0.148
(2)		−	−	0.042
B		+	−	0.095
VI. Dilute phenol extracted brucella				
A (1a)		+	−	0.046
(1b)		Weakly +	−	
B (1a)		+	+	
(1b)		Weakly +	−	0.038
(2a)		Weakly +	−	0.059
(2b)		Unsatisfactory	Unsatisfactory	

+ positive reaction.
− no reaction.
* Refers to outline in text.

would be related to the same antigenic fraction precipitated in direct contact with immune serum. In the early studies only those extracts were considered suitable which gave positive precipitation reactions. It was soon found, however, that there was no relationship between the precipitated antigen and the adsorbed antigen. These findings are shown in table 2.

The first 28 extracts were tested according to the method of Middlebrook and Dubos (1948). Negative results were obtained, and none of these extracts could be shown to be adsorbed onto sheep cells, using their method. There was absolutely no indication of any agglutination of the supposedly sensitized sheep cells which had been treated with those extracts which were precipitated by immune serum. Tests of the supernatant solution from the agglutination tubes showed the precipitating substance to be present. Apparently little or no adsorption of this fraction onto sheep cells had taken place.

At about this time Boyden's work (1951) appeared dealing with the treatment of erythrocytes with tannic acid and their subsequent hemagglutination by antiprotein serums. Attempts to apply this technique and to sensitize tannic acid-treated sheep cells with brucella extracts were completely negative.

A third series of experiments were performed in which these two types of tests were repeated, using normal rabbit serum and crystalline bovine albumin

TABLE 3.—*Results of serums from known or suspected cases of brucellosis tested with adsorbed antigens.*

Specimen	Remarks	Screen test†	Standard test‡		Brucella extract VIA(1a) adsorbed onto collodion							
			Plate	Test tube	Dilution of patients serum							
					1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
F.J.	Vaccine therapy	+	1:80 slow	1:80	4§	4	4	3	2	—	—	—
C.M.	Vaccine therapy	+	1:160 slow	1:80	1	2	2	2	—	—	—	—
A.C.	Arthritis; positive chest plate but negative sputum and gastric washings for T.B.	+	—	1:40	4	3	2	—	—	—	—	+
J.L.	Positive skin test, clinically positive	±	1:320	1:160	2	2	1	1	—	—	—	—
J.D.S.	Positive skin test, clinically positive	±	1:320	1:80	3	3	2	2	—	—	—	—
M.F. 16	No clinical evidence; negative skin test	±	—		4	1	—	—	—	—	—	—
L.F. 19	Raised on farm; negative skin test	±	—		4	4	—	—	—	—	—	—
J.W.A. 85	Check on progress	+	1:320	1:640	4	4	3	3	2	—	—	—
CBF 10	Farmer	+	1:320	1:640	4	4	2	2	—	—	—	—
A.L.B. 92	Vaccine therapy	+	1:320	1:640	4	4	4	3	3	2	—	—
H.M.S.	Clinically positive	+	—	partial 1:80	4	3	3	1	1	—	—	—
H.H.B.	Clinically positive	±	1:160	1:80		2	2	3	3	1	—	—
C 63	Abortus 1:40	+	1:80 slow	1:80	2	2	2	2	—	—	—	—
C 58	Abortus 1:160	+	1:320	1:320	4	4	2	2	1	—	—	—
C 4*	Melitensis 1:1280, abortus 1:640	±	zone 1:320	1:160	—	—	—	3	2	—	—	—
C 35*	Abortus 1:40	+	—	—	3	—	—	1	2	3	3	—
C 1-X*	Abortus 1:320	±	zone 1:320	zone 1:160	2	1	—	—	—	—	—	—
C 69*	Abortus 1:160	+	1:40	1:40	2	3	4	4	4	4	—	—
10 specimens	No clinical findings	—	—	—	—	—	—	—	—	—	—	—

* 50% citrated plasma.
† Screen test has been previously described (Hirschberg, 1950).
‡ Standard tests were performed with *Br. abortus* #1119 (see ref. 7). Figures indicate highest dilution showing agglutination.
§ Figures indicate degree of agglutination (cf. text).

as diluents as described by Boyden (1951), Griffitts (1947), and Milzer and Nathan (1947). The results from these experiments were also completely negative.

It was then considered that since certain bacterial extracts could be adsorbed onto sheep cells and not onto collodion, perhaps other substances might be adsorbed onto collodion which apparently would not sensitize sheep cells. In these experiments 9 extracts of brucella previously negative with sheep cells were adsorbed onto collodion with ease, strongly agglutinated by immune serum and 5 weakly agglutinated. Only 1 extract adsorbed onto collodion was agglutinated by negative serum, i.e., gave a falsely positive reaction. This extract was discarded.

When centrifugation proved to be the method of choice for the reading of the tests, two out of three of the extracts which were positive with collodion were found to sensitize sheep cells strongly and one weakly. Five extracts remained uusatisfactory for the sensitization of sheep cells. Although iso-

tonic solutions, they contained fractions of brucella which hemolyzed sheep cells.

Table 2 shows the results obtained with various brucella extracts. The extracts are divided into groups according to the first step in the production of the extract. The results of the precipitation test are included for purposes of comparison only. Also included in the table are some figures on the nitrogen content of the extracts. It is obvious that there is no relationship between the nitrogen content and the ability of the extracts to be adsorbed onto collodion or to sensitize sheep cells. The extracts have been concentrated, and results of chemical tests of the concentrated extracts will be made in a future communication.

From table 2 it will be seen that the best antigens are IIC(2), IID(3) and VIB(1a). IIC(2) is the extract prepared by treating ground brucella with $N/2$ trichloracetic acid, redissolving and concentrating the small amount of protein obtained. IID(3) is the dialyzed supernatant trichloracetic acid, presumably protein free, but actually showing a small amount of nitrogen. VIB(1a) is the extract prepared by 30-day extraction of brucella with 2% neutral phenol.

All three of these extracts could be adsorbed onto both sheep cells and collodion, resulting in antigens which were agglutinated strongly and in high dilution by immune brucella serum. No false positive nonspecific agglutinations were obtained with negative serums.

Antigen VIA(1a) did not sensitize sheep cells but was adsorbed onto collodion and resulted in an antigen which was strongly agglutinated by immune brucella serums. This antigen was used to test the serums from 18 suspected or proven cases of brucellosis. The results of these experimental tests compared with standard tests are shown in table 3. None of these 18 serums failed to react with antigen VIA(1a) in the presence of a positive standard test. Four serums agglutinated adsorbed antigen VIA(1a) when the standard plate test was negative. In 3 of these 4 cases, these findings led the way to a clinical diagnosis of brucellosis. The 4th case was not confirmed clinically. Blood cultures on all 18 were negative.

Antigen VIA(1a) was not agglutinated by 8 serums from known cases of tularemia with high agglutinin titers against *Past. tularense*, showing a high degree of specificity.

SUMMARY

1. Extracts have been prepared from brucella, which when adsorbed onto collodion, yield antigens for the agglutination tests sensitive and specific for the detection of brucella immune substances in serum. A method is reported for the simple production of a stable collodion suspension.

2. Some of the extracts sensitized sheep erythrocytes to a limited degree and are suitable for the hemagglutination test for the detection of brucella immune substances in serum. It is possible that appropriate concentration may yield more sensitive extracts.

3. Preliminary findings in the use of the extracts for the diagnosis of clinical brucellosis are reported. The tests appear to be highly specific. Further studies on the relative value of the quantitative and qualitative tests with adsorbed antigens are underway.

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STUDIES ON STREPTOCOCCI

IV. A STUDY OF THE RELATIONSHIP OF CERTAIN VIRULENCE FACTORS IN STREPTOCOCCAL INFECTIONS TO THE LD_{50} DOSE OF STREPTOCOCCI

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In the first paper of this series, Sherwood, Wahl, Hamilton, Colglazier and Jay[1] reported upon experimental infection of the chorioallantoic (C.A.) membrane of the embryonic chick by 34 strains of β hemolytic streptococci, one strain of α', and one strain of *Streptococcus viridans*. They found all strains could cause an inflammatory reaction and more or less necrosis of the ectoderm, but only eight strains, two group B, five group D, and one of group K, invaded and spread to a greater or less degree in the mesoderm. Russell and Sherwood[2] showed that only two of these produced hyaluronidase in vitro. They observed no correlation between virulence and the ability to spread within the mesoderm nor any correlation between virulence and any particular Lancefield group. While a great deal has been written about the relationship of hyaluronidase, fibrinolysins, leucotoxins, oxygen-labile hemolysins, hyaluronic acid formation, invasiveness of the mucous membrane and apparent generation time to virulence of streptococci for mice and man, there have been, except for the work of Russell and Sherwood[2] on hyaluronidase, no reports on the relationship of these to virulence for the embryonic chick. The latter is an especially interesting experimental animal because, in many instances, it reflects phenomena observed in human infection not observed in mice or other experimental animals. Goodpasture and Anderson[3] brought this out in their study on infections of the C.A. membrane by various types of bacteria. It is of interest to note that they, as well as Sherwood et al,[1] found strains of streptococci from cases of subacute endocarditis were physiologically different from groups A and C streptococci in that the endocarditis streptococci could establish themselves in cells of mesodermal origin as host cells. The endocarditis streptococcus employed by Goodpasture and Anderson[3] was *Str. viridans*, while those employed by Sherwood et al,[1] were β hemolytic ones belonging to Lancefield groups B, D, and K, respectively, and one strain of *Str. viridans*. The embryonic chick has also been found to be of great value in the study of many viral and rickettsial infections.

Most of the studies of virulence factors of streptococci for mice have dealt with the factors individually rather than collectively, and no study of their collective relationship for the chick has

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2. Russell, B. E. and Sherwood, N. P. 1949, The role of hyaluronidase in experimental streptococcal infection. J. Infect. Dis. **84**: 81–87.
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been made; and virulence for the mouse has, with few exceptions, been expressed in terms other than the LD_{50} dose. Therefore, it seemed desirable to attempt to answer the following questions by experiment:

1. What is the relative virulence in terms of the LD_{50} dose for the mouse and chick of 23 strains of β hemolytic streptococci from human infections and two others from infections in guinea pigs and the horse, respectively?

2. How does the relative invasive power of these streptococci for the mucous membrane of the mouse and the C.A. membrane of the chick compare?

3. How does hyaluronidase production in vitro compare with the production of skin spreading factors in vivo?

4. Is there a difference in the physiological effect of supernatants on human neutrophiles, mouse neutrophiles, and mouse monocytes?

5. Is there a correlation between oxygen labile hemolysin and leucocidin?

6. Is there either individually or collectively a correlation between the LD_{50} for the mouse or the chick and spreading factors, leucocidins, fibrinolysins, apparen generation time and oxygen labile hemolysins?

The answers to these questions might indicate whether one or a combination of some or all of these factors play a major role in fatal streptococcal infection or whether some other factor or factors produced within the body as a result of the interaction of host and parasite are the important ones in producing profound illness or death of the host.

MATERIALS AND METHODS

The LD_{50} or 50% end points were determined by the method of Reed and Muench.[4]

Eggs.—Eggs containing 13-day-old viable embryos were employed. These eggs were from pure bred white leghorn hens.

Mice.—Mice were from an inbred strain of white mice obtained from the Maple Grove Rabbitry, Springfield, Missouri. The weights were from 18 to 20 g.

Bacteria.—The Lancefield grouping and source of the 25 strains of streptococci are given in table 1.

Culture mediums.—Except for studies of streptolysin O, only two types of mediums were employed. One was the tryptose phosphate horse serum medium previously employed by Sherwood et al,[1] and the second was a proteose peptone #2 broth with a pH of 7.3.

Immune serums.—Grouping serums were obtained from the Communicable Disease Center at Chamblee, Georgia.

Dosage.—For the C.A. membrane of the chick and the intraperitoneal injections of the mouse, 0.02 and 0.5 ml, respectively, of dilutions of 24-hour culture ranging from 1 to 10^{-6} were employed. For intranasal inoculations of the mouse, 0.05 ml was the dose.

Technique.—The egg technique described by Goodpasture[5] was employed in the embryonic chick studies. To determine the LD_{50} dose, six eggs were inoculated with each dilution. In determining the LD^{50} dose for mice, two series of six mice were injected, at different times, intraperitoneally with 0.5 ml of each dilution. Intranasal inoculations of ten mice with 0.05 ml of each culture were accomplished by employing light ether anesthesia and using a tuberculin syringe and 26-gauge needle. A group of ten uninoculated mice were kept as controls.

Hyaluronidase.—The methods of hyaluronidase determinations were those employed by Russell and Sherwood.[2]

Skin spreading factors.—The technique consisted of the intradermal injection of solutions containing 0.45 ml of culture filtrate and 0.05 ml of an indicator solution or suspension into rabbits. Controls using sterile broth and also standardized testicular extracts containing an indicator were included. The indicators used were India Ink, trypan blue, hemoglobin and T. 1824 or Evans blue. Since India Ink and trypan blue were rather difficult to see under the epidermis and trypan blue has some anticoagulant action which might affect the response, it seemed advisable to try the dye T. 1824. A 10% aqueous

4. Reed, S. J. and Muench, H. 1938, A simple method of estimating fifty percent end points. Am. J. Hyg. **27**: 493–497.

5. Goodpasture, E. W. 1933, Use of embryo chick in investigation of certain pathological problems. South. M. J. **26**: 418–420.

solution was prepared. The results were quite satisfactory.

Skin reactions.—The long and short diameters of the area of spread were measured 24 hours after the injection. The areas of spread were calculated using the formula for an ellipse as follows: Area of spread $= \pi Dd/4$, where D and d represent the lengths of the long and short diameters, respectively. Indices were calculated as suggested by Claude.[6] The area of spread of the test filtrate is divided by the area of spread of a control solution of sterile, uninoculated liquid medium used. Claude considered an index of 1.4 or more as positive.

Leucocidin index.—One-tenth ml of citrated blood was mixed with 0.05 ml of the supernatant from 8- and 18-hour broth cultures, respectively, of each streptococcus, and a corresponding amount of the same blood with 0.05 ml of the sterile broth was employed as a control. The pH was adjusted to 7.3. These mixtures were incubated for 30 minutes at 37 C, and 0.05 ml of a bacterial suspension added and the tubes incubated with frequent shaking for 30 minutes. The index was calculated by dividing the number of bacteria phagocytized by 25 leucocytes in blood containing a supernatant by the number phagocytized by leucocytes in blood to which broth was substituted for the supernatant. The experiments were repeated several times with quite consistent results. Two types of leucocytes, human and mouse, and two kinds of bacteria, brucella and streptococci, were used in this work.

Human blood from a case of chronic brucellosis was used to determine the effect of streptococcus supernatants on the phagocytosis of *Brucella abortus.*

Pooled normal mouse blood was used to determine the effect of streptococcus supernatants on the phagocytosis of sensitized suspensions of a strain of streptococcus used to immunize goats.

Streptokinase (fibrinolysins).—A meat infusion broth containing 0.05% glucose, pH 7.6, as recommended by Tillett and Garner,[7] was used for the routine test. The cultures were grown at 37 C for 18 hours. The technique of the test consisted in adding 0.8 ml of saline to 0.2 ml of oxylated plasma (0.02 g per 10 ml of blood). To the plasma saline solution, 0.5 ml of whole culture was added, followed immediately by the addition of 0.25 ml of 0.25% calcium chloride solution The tubes were mixed and incubated at 37 C in a water bath. The time of clotting was observed (this occurred in approximately 5 minutes). The tubes were then watched closely and the time of lysis was noted. If no lysis occurred within two hours, the results were recorded as negative.

Apparent generation time.—The technique employed was one described by Lewandowski and Stahly.[8] The culture medium was tryptose phosphate broth containing 0.2% glucose and 5% horse serum. Growth was measured by measuring the optical density of the culture at frequent intervals, using a Coleman model 14 spectrophotometer. A wave length of 600 mμ was used in all the determinations. The average of three optical density readings taken at one time was recorded. The mean value was plotted against time, using semilogarithmic graph paper. The apparent growth rates were calculated from the straight line portion of the curve according to the following formula:

$$g = \frac{t \cdot \log 2}{\log b - \log B}$$

where g = apparent generation time, t = time interval, b = density at the top of the straight line portion of the curve, and B = density at the bottom of the straight line portion of the curve.

Oxygen-labile hemolysin.—Two types of mediums were employed in the production of streptolysin O. One of these was Swift and Hodge's[9] modification of the Todd-Hewitt[10] medium. The second medium was found quite satisfactory and consisted of the following:

proteose peptone #3	20 g
yeast extract	6 g
dextrose	2 g
sodium chloride	5 g
disodium phosphate	5 g
distilled water	1000 ml

The pH of the above medium was adjusted to 7.8.

6. Claude, A. 1937, Spreading properties of leach extracts and the formation of lymph, J. Exper. Med. **66**: 353–366.
7. Tillet, W. S. and Garner, R. L. 1933, The fibrinolytic activity of hemolytic streptococci. J. Exper. Med. **58**: 485–502.
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10. Todd, E. W. and Hewitt, L. F. 1932, A new culture medium for the production of antigenic streptococcal hemolysin. J. Path. & Bact. **35**: 973–974.

Reducing agent.—The standard reducing agent employed was a solution of sodium hydrosulfite in alkaline isotonic phosphate buffer. A concentration of 7.5 mg/ml was employed. Reduction was effected by mixing the culture supernatants, after centrifugation at 5000 revolutions per minute, for 20 minutes with an equal volume of reducing agent and allowing the supernatants to stand in the cold at 6 C for 15 minutes aerobically.

Hemolysin test.—After the incubation period the cultures were quickly frozen, centrifuged while frozen (the cultures thawed out during the centrifugation process) and the supernatants collected and reduced. Twofold dilutions of the supernatants in physiological saline were made, beginning with a dilution of 1:2. The mixture of equal volumes of reducing agent and lysin was considered to be a 1:2 dilution. Diluted lysin, 0.5 ml, was mixed with 0.5 ml of a 5% suspension of sheep erythrocytes thrice washed in physiological saline. The tubes were incubated in the 37 C water bath for 30 minutes. At the end of this time the tubes were centrifuged if necessary and read. In no instance did the hemolytic titer exceed 1:32; hence this was the highest dilution employed.

RESULTS

Relative virulence for the mouse and chick.—From an inspection of table 1, it will be seen that none of the strains in any of the Lancefield groups studied were avirulent for the chick. Two group A strains were slightly virulent (LD_{50} 10). One group A and one group D showed moderate virulence since the LD_{50} was $10^{-0.4}$ and 10^{-1}, respectively, while the rest of the 25 strains had an LD_{50} of $10^{-2.5}$ to 10^{-4}. There was thus some correlation between ability to infect man, horse, and guinea pig, and virulence for the chick. The avirulence for the mouse of two strains of group A and two of group D, all from clinical cases in man, shows a definite lack of value of the mouse as a test animal for determining virulence of streptococci for man. It is also evident from an inspection of table 1 that the mucous membrane resistance of the mouse is much greater than the barrier resistance of the C.A. membrane of the chick. Judged by the LD_{50} dose only two strains of group A and two of group C strains showed any appreciable invasive power of the mucous membrane of the mouse, while all 25 strains showed definite invasive powers for the C.A. membrane of the chick. In an attempt to obtain experimental data bearing upon this question, heart blood cultures were made on apparently healthy survivors of mice ten days after recieving intranasal inoculations of five group A, two group B and three group C streptococci. Positive blood cultures were obtained from one or more survivors of three group A, both of the group B and one of three group C inoculations. The LD_{50} apparently differentiates only between lethal invasion and either partial or no invasion.

Relation of hyaluronidase and spreading factor to virulence.—It is obvious from an inspection of the data in table 1 that there is no correlation between virulence for the chick, mouse, or man and the production of hyaluronidase in vitro or the spreading factor in rabbits. Group A strain 1061, from a case of erysipelis, group D strain 1190, from a serious case of infection, and strains 1081 of group C produce neither of those factors, but are virulent for both mouse and chick, while strain 1063, from a case of tonsillitis, is avirulent for the mouse, virulent for the chick and produces both factors. It is interesting to note that group A strains 1068 and 1072 and group C strains 1059 and 1081 showed marked ability to invade the mucous membrane of the mouse, although neither produced hyaluronidase in vitro.

Leucotoxins and virulence.—From an inspection of table 1, it will be seen that there is no correlation between the effect of streptococcal supernatants on either human or mouse leucocytes and their virulence for mice. The supernatant of group A strain 1063 gave a leucocidin

TABLE 1.—*A comparison of virulence factors with the LD_{50} for the mouse and chick.*

Group	Strain and group	Mouse virulence		Chick LD_{50}, 0.02 ml	Hyaluronidase	Spreading factor	Leucocidin index			Fibrinolysin (streptokinase)			Apparent generation time (min.)	Oxygen labile hemolysin	Source
		I.P. LD_{50}, 0.5 ml	I.N. mortality, 0.05 ml				Man P	Mouse P	Mouse M	Human	Mouse	Chicken			
A	1040	10	10%	10^{-3}	−	−		0.34	4.3	−	−	−	28.2	−	Tonsillitis
	1042	avirulent	0	1	−	+		0.3	0.23	+	−	−	28	−	Tonsillitis
	1061	10^{-1}	0	10^{-3}	−	−		0.23	0.45	+	−	−	38	−	Erysipelas
	1063	avirulent	0	10^{-3}	+	+		0.3	0.45	+	−	−	17.4	+	Tonsillitis
	1066	10	0	1	−	−		0.3	0.68	+	−	−	33.5	−	Pus, human
	1068	10	40	$10^{-0.4}$	−		0.62	0.45	2.72	+	−	−	82.0	+	Murray
	1070	$10^{-0.4}$	10	10^{-2}	−	+	0.92	0.53	0.45	−	−	−	49.5	+	Tonsillitis (Rheumatic fever)
	1071	10	0		−	+	1.02	0.74	3.58	+	−	−	79.5	−	Tonsillitis (Rheumatic fever)
	1072	10^{-1}	50	10^{-3}	−	−		0.3	0.9	+	−	−	50	−	Tonsillitis (Rheumatic fever)
	1073	10	0		−	−	0.69	0.3	0.23	+	−	−		−	Tonsillitis (Rheumatic fever)
B	1076	10^{-14}	0	10^{-3}	+	+	0.47	0.53	1.59	−	−	−	27	−	Kidney infection
	1079	10^{-2}	0	10^{-2}	+	+	0.68	0.15	0.0	−	−	−	70.2	−	Cervix
C	1059	$10^{-0.4}$	40	10^{-2}	+	+		0.23	0.45	+	−	−	20	−	G.P. septicemia (Epidemic)
	1081	10^{-2}	70	10^{-2}	−	−		0.78	5.7	+	−	−	56.9	−	G.P. septicemia (Epidemic)
	1082	10^{-2}	0	10^{-3}	+	+		1.56	3.58	+	−	−	43.2	−	Tonsillitis (human)
	1083	10^{-2}	0	10^{-2}	±	+		0.45	2.86	−	−	−	51.4	−	Infection in a horse
	1084	10^{-1}	20	10^{-2}	−	+		0.45	0.45	+	−	−	31	−	Tonsillitis
	1085	10	0	10^{-2}	−	+		0.23	0.9	+	−	−	26.5	−	Mouth Infection
D	1008	10	10	10^{-1}	−	+		0.43	1.46	−	−	−	30.5	−	Endocarditis
	1017	10	0	10^{-2}	−	+	0.58	1.59	8.56	−	−	−	28.8	−	Endocarditis
	1090	avirulent	0	10^{-2}	−	+		1.14	1.43	−	−	−	29.5	−	Tonsillitis
	1190	$10^{-2.5}$	10	10^{-2}	−	−	0.57	0.3	0.45	−	−	−	24	−	Endocarditis
	1191	10	0	10^{-2}	−	+		0.36		−	−	−	28.1	−	Tonsillitis
	1192	avirulent	20	10^{-2}	−					−	−	−	28.9	−	Tonsillitis
	1193	avirulent	0	$10^{-2.5}$	−	±		0.15	0.45	−	−	−	28.8	−	Human Infection
Controls			0	0											Broth ph 7.3

index of 0.3 for mouse polymorphonuclear cells and 0.68 for mouse monocytes and was avirulent for mice. On the other hand, strain 1076 group B supernatant gave a leucocidin index of 0.53 for the neutrophiles and 1.59 for mouse monocytes and had an LD_{50} of $10^{-1.4}$ for the mouse. Streptococcus 1193, belonging to group D, produced a supernatant that gave a leucocidin index of 0.15 for the mouse neutrophiles and 0.45 for monocytes and was avirulent for the mouse. It is only moderately virulent for man. The increased rather than decreased phagocytosis of streptococci by mouse monocytes produced by the supernatants of group A streptococcus 1068 and group B streptococcus 1076 is interesting. These supernatants seem to stimulate phagocytosis by the monocytes. Since monocytes are regarded as important in the body's defense against streptococci, such substances, if produced in vivo by these streptococci or contaminating organisms or secondary invaders in a wound, would favor phagocytosis of such streptococci. Group A strain 1068 was moderately virulent for the mouse and the chick, which correlates quite well with a leucocidin index of 0.45 for mouse neutrophiles, but not with the leucocidin index of 2.72 for mouse mononuclears.

Fibrinolysins (streptokinase).—An inspection of table 1 shows that only two of the ten group A strains failed to produce streptokinase for human fibrin. One of these two was from a case of tonsillitis in a rheumatic fever patient. None of the ten strains produced lysis of mouse or chicken fibrin, although only two were avirulent for the mouse and all were virulent for the chick. None of groups B and D streptococci from clinical cases produced streptokinase. Group C streptococci, all highly virulent for both the mouse and the chick, did not produce streptokinase for mouse or chicken fibrin, although five of the six strains produced a streptokinase for human fibrin.

Apparent generation time and virulence.—Lack of correlation between virulence and apparent generation time in vitro is indicated by the following: Group A strains 1040 and 1068 had apparent generation times of 28.2 minutes and 82 minutes, respectively. Both had the same LD_{50} for the mouse, while the former had an LD_{50} of 10^{-3} and the latter $10^{-0.4}$ for the chick. Group A strain 1072 was fairly virulent for the mouse, quite virulent for the chick, and had an intermediate apparent generation time of 50 minutes. In group B, two had an LD_{50} of 10^{-3} for the chick and $10^{-1.4}$ and 10^{-3}, respectively, for the mouse. The more virulent of the two for the mouse and chick had a long apparent generation time. Definite inconsistencies also hold for strains 1084 and 1085 of group C, since 1084, the more virulent of the two for the mouse, had a longer apparent generation time than 1085, while both have the same LD_{50} for the chick.

Oxygen-labile hemolysins and virulence.—It is interesting to note that group A strain 1063, producing an oxygen-labile hemolysin, was avirulent for mouse and highly virulent for the chick, while strain 1066 was moderately virulent for both the mouse and the chick but did not produce an oxygen-labile hemolysin. Group A strain 1068 produced an oxygen-labile hemolysin, and was moderately virulent for both the mouse and chick, while group A strain 1071, that did not produce the hemolysin, was more virulent for the same hosts. In group C, strains 1082 and 1083 were highly virulent for the mouse and for the chick and neither produced an oxygen-labile hemolysin.

DISCUSSION

The literature contains many papers dealing with virulence of streptococci

for the white mouse, but apparently the only ones using the embyonic chick are those by Goodpasture and Anderson,[3] and Sherwood, Wahl, Hamilton, Colglazier and Jay.[1] It is difficult to evaluate the data in the literature on virulence of streptococci for the mouse, because of the various techniques employed. Cowan[11] determined virulence by injecting each mouse with all the streptococci obtained from one blood agar slant. Tunnicliff[12] determined the virulence of streptococci by injecting the washings of a 24-hour chocolate agar plate culture intraperitoneally into a mouse. Delves[13] tested the virulence of an S type streptococcus by injecting intraperitoneally into a mouse the growth from a 24-hour blood agar slant suspended in saline. To test the virulence of R colony type streptococci, she employed suspensions of from one to three blood agar slants. Cooley[14] employed the growth of one to three blood agar slants in determining the virulence of streptococci for mice. Dawson, Hobby and Olmstead[15] used smaller doses in determining virulence, since they say that the R variant failed to kill mice in amounts of culture less than 0.10 ml, while the original culture killed in a dilution of 10^{-6}. Pike[16] employed 0.2 ml of broth dilutions of cultures grown for four hours in rabbit blood infusion broth. He found no correlation between hyaluronic acid production and virulence for mice. It will be observed from an inspection of table 1, that virulence for the mouse following intraperitoneal injection and of the chick inoculated on the C.A. membrane, is expressed by the LD_{50} dose. Virulence for mice inoculated intranasally is expressed in terms of percentage mortality.

All but a few of the cultures were obtained from clinical cases in man. The data in table 1 show no correlation between virulence for the mouse and that for the chick. There is a good correlation between the virulence for the chick and the ability to infect and produce inflammatory reaction in man. Group C strains 1059 and 1081 were highly virulent for the guinea pig, and strain 1083 produced severe infection in a horse. It must be realized, however, that the use of the methods for determining virulence, including the LD_{50} dose, are open to the same criticism made by Theobald Smith[17] to virulence studies in tuberculosis. He pointed out that in natural infection with the tubercle bacillus, the entry through the membrane of the respiratory or alimentary tract is not accompanied by mechanical injury to the tissue, such as results from hypodermic injection, and the dosage is limited to one or at most a few organisms, as contrasted with relatively massive doses employed in experimental work. Furthermore, the organisms involved in natural infection have a rate of multiplication and a metabolism that show the effect of a parasitic environment, whereas in experimental work, the organisms have been acclimated to a saprophytic existence in

11. Cowan, M. 1922, Variation phenomena in streptococci with special reference to colony form, hemolysin production, and virulence. Brit. J. Exper. Path. **3**: 187–195.
12. Tunnicliff, R. 1931, The various colonies of hemolytic streptococci in scarlet fever. J. Infect. Dis. **49**: 357–365.
13. Delves, E. 1932, Dissociation in Streptococcus scarlatinae. J. Infect. Dis. **50**: 350–357.
14. Cooley, L. E. 1932, Dissociation of streptococci. J. Infect. Dis. **50**: 358–366.
15. Dawson, M. H., Hobby, G. L. and Olmstead, M. 1938, Variation in the hemolytic streptococci. J. Infect. Dis. **62**: 138–168.
16. Pike, R. M. 1948, Streptococcal hyaluronic acid and hyaluronidase. III. Virulence of group A streptococci for mice in relation to the production and destruction of hyaluronic acid. J. Infect. Dis. **83**: 19–22.
17. Smith, T. 1933, Focal cell reactions in tuberculosis and allied diseases. Johns Hopkins Hosp. Bull. **53**: 197–225.

artificial mediums and possess an accelerated rate of growth. Their metabolic products are essentially different from those of organisms that have been growing under parasitic conditions. While Smith's criticisms are justified, experience has shown that virulence studies in which cultures are employed are not without merit. The data presented in table 1 not only indicate clearly marked difference in virulence of various strains for the mouse when introduced intraperitoneally, but show striking differences in their ability to produce respiratory infection in the mouse and to invade the C.A. membrane of the chick. By experiments not reported in the table, it was found that rapid transfer into proteose peptone #2 broth of most of these streptococci during the logarithmic stage markedly increased their virulence for the mouse and the chick. In considering the virulence of these streptococci for man, there is some incongruity in comparing LD_{50} for the mouse and the chick with only clinical conditions such as tonsillitis, erysipelas and abscess formation in man or clinical evidence of infection in other animals. However, three of the strains, all belonging to group D, were from fatal cases of subacute bacterial endocarditis. Two group C strains were from fatal cases of streptococcal infections occurring in guinea pigs.

In previous communications, Sherwood et al[1] and Russell and Sherwood[2] review the literature bearing upon the role of the spreading factors discovered by Duran-Reynals[18] and of hyaluronidase production to virulence of bacteria for various hosts. They offer experimental evidence indicating that neither the ability of streptococci to spread within the mesoderm nor their ability to produce hyaluronidase in vitro can be correlated with their virulence for the embryonic chick. The data in table 1 not only confirm these conclusions, but indicate a similar lack of correlation of these factors with virulence of streptococci for the white mouse when the route of inoculation is either intraperitoneal or intranasal. Since Duran-Reynals,[18] McClean[19] and others have shown that spreading factors are able to overcome the first physiological barrier encountered by invading organisms and play a role in the spreading of the infectious agent in the tissues, it is obvious that, while they are not lethal factors, they are quite important in the establishment of virulent organisms in the body. Duran-Reynals[18] considers two possible roles of the spreading factor. It may be instrumental in the spread of infection or it may cause such an extreme dispersion of the bacteria that at no place are there enough bacteria to set up infection.

Swift[20] states that "most experimental data apparently exclude any direct relationship between streptococcus virulence and hyaluronidase production." He cites the work of Thompson[21] and suggests, however, that it is "hypothetically conceivable that under as yet unknown circumstances this enzyme might attack the somatic hyaluronic acid focally and thus induce lesions."

Other physiological products of some

18. Duran-Reynals, F. 1928. Exaltation de l'activite du virus vaccinal par les extraits decertains organes, Compt. rend. Soc. de biol. **99**: 6–7. See also: Duran-Reynals, F. 1942, Tissue permeability and spreading factor in infections. Bact. Rev. **6**: 197–251.

19. McClean, D. 1930, The influence of testicular extract on dermal permeability and the response to vaccine virus. J. Path. & Bact. **33**: 1045–1070.

20. Swift, H. F. 1948, Bacterial and Mycotic Infections of Man, Philadelphia, J. B. Lippincott Co., 237–290.

21. Cited by Swift (see above).

but not all streptococci are substances that either destroy phagocytic cells or interfere with phagocytosis. The destructive action of bacteria on leucocytes has been recognized for many years and was first described as a property of staphylococcus toxin. Ruediger[22] observed that filtrates of virulent hemolytic streptococci inhibited the phagocytosis of nonvirulent streptococci. The factors in supernatants toxic for leucocytes are generally called leucocidins or leucotoxins.

McLeod[23] observed that hemolytic organisms produced leucocidins and stated that there is a definite relationship between virulence and the ability of an organism to produce leucocidin. Nakayama[24] used the methylene blue reduction technique, introduced earlier by Neiser and Wechsberg in their studies on staphylococcal leucocidins, to study leucocidin production by streptococci. Nakayama found that, in general, hemolytic and nonhemolytic streptococci, when nonvirulent, did not produce any leucocidin as demonstrated by the reduction test; however, virulent strains produced definite leucocidins. He noted that streptococci formed the greatest quantity of leucocidin after from 10 to 24 hours, and he also observed that there was a reduction in leucocidin content of a culture after 48 hours. Antistreptoleucocidin did not neutralize staphyloleucocidin. He stated also that streptoleucocidin was distinct from hemolysin.

Channon and McLeod[25] studied the relationship of leucocidin to erythrogenic toxin and concluded that leucocidin was distinctive from skin toxin but that hemolysin and leucocidin were probably identical. Evans[26] observed that leucocidin was distinct from both hemolysin and erythrogenic toxin. Gay and Oram[27] concluded from their studies that streptococcus leucocidin was distinct from hemolysin because (1) leucocidin was formed earlier in the culture, (2) it had the ability to remain in the filtrate for a longer period, and (3) it was more thermostable. The results reported in table 1 are in agreement with the conclusions of Evans[26] and also of Gay and Oram.[27] These workers made several other interesting observations. They found that the leucocytes of dogs and guinea pigs were not susceptible to the leucocidins which they were using, although the same leucocidins destroyed the leucocytes of the rabbit. They also found that rabbit clasmatocytes were quite resistant to leucocidin, while the polymorphonuclear cells were easily affected. They were able to produce antistreptoleucocidin only by using as an antigen the pleural exudate of rabbits dead of infection.

It is evident from an inspection of the data in table 1 that the phagocytosis by mouse neutrophiles of sensitized streptococci was definitely reduced by the supernatants of 19 of 24 streptococci belonging to groups A, B, C, and D that were tested. On the other hand,

22. Ruediger, G. J. 1903, Production and nature of streptolysin. J. A. M. A. **41**: 962–964.
23. McLeod, J. W. 1914, Criteria of virulence amongst streptococci with some remarks on streptococcal leucocidin. J. Path. & Bact. **19**: 392–413.
24. Nakayama, Y. 1920, On the toxin for leucocytes produced by streptococci (streptoleukocidin). J. Infect. Dis. **27**: 86–100.
25. Channon, H. A. and McLeod, J. W. 1929, On the importance of thermolabile streptococcal toxin, with special reference to its cytolytic effect on leucocytes. J. Path. & Bact. **32**: 283–291.
26. Evans, A. C. 1931, The effect of hemolytic streptococci and their products on leucocytes. Pub. Health Rept. **46**: 2539–2557.
27. Gay, F. P. and Oram, F. 1933, Streptococcus leucocidin. J. Immunol. **25**: 501–525.

a marked reduction in phagocytosis by mouse monocytes was caused by only 10 of 23 supernatants. Ten of the supernatants, three of group A, one of group B, three of group C and three of group D, apparently enhanced phagocytosis. Theoretically, if this occurred in the mouse and if the monocytes are important in host defense, it should be reflected by decreased virulence. The results suggest that leucotoxins for mouse leucocytes are not formed in vitro by some streptococci, and that either the monocytes are not of major importance in protecting the mouse or that the phenomenon does not occur in vivo. The results also suggest that mouse leucocytes were more susceptible to injury by the leucocidins than human neutrophiles.

The fact that the data in table 1 show no correlation between the leucocidin index and virulence for man, mouse, or chick is not interpreted as indicating that leucocidins are of no importance in infection. The results are regarded as showing that while leucocidins can and do injure or impair the function of certain important cells of the body, they alone are not the lethal factors in infection. If all leucotoxins are oxygen-labile, as Todd[28] reports, it is difficult to explain the results appearing in the mixture of blood and supernatant which were obtained without the addition of a special reducing agent, as is necessary for streptolysin O experiments.

Another physiological property that has been suggested as impotant in virulence is the ability of broth cultures and filtrates of hemolytic streptococci to liquefy clotted human plasma. This property was discovered by Tillet and Garner.[7,29,30] They found that plasma clots of about 75% of normal individuals were susceptible to streptococcal fibrinolysin. Twenty-five percent of these clots showed varying degrees of resistance to lysis. Later work has shown that this resistance occurs commonly in patients with various streptococcal infections.

Hadfield, Magee and Perry[31] found that the average virulence for mice of their strains, that were most potent in the production of fibrinolysin for human fibrin, was higher than that of the least active.

The specificity of the fibrinolysic activity with respect to the source of the plasma merits some consideration. Tillett and Garner[7] reported that strains of streptoccoci which were able to lyse normal human fibrin clot were not able to lyse normal rabbit fibrin clot. Madison[32] investigated the fibrinolytic activity of 12 strains of hemolytic streptococci from infections in horses against fibrin from horse, man, hog, cow, and rabbit. These equine strains liquefied horse fibrin but did not have an effect on the fibrins derived from other species. Smith et al[33] found that 9 of 22 strains of hemolytic streptococci isolated from cow's milk had the ability to lyse bovine

28. Todd, E. W. 1942, The leucocidin of group A haemolytic streptococci. Brit. J. Exper. Path. **23**: 136–145.

29. Garner, R. L. and Tillett, W. S. 1934, Biochemical studies on the fibrinolytic activity of hemolytic streptococci. I. J. Exper. Med. **60**: 239–254.

30. Garner, R. L. and Tillett, W. S. 1934, Biochemical studies on the fibrinolytic activity of hemolytic streptococci. II. Nature of the reaction. J. Exper. Med. **60**: 255–267.

31. Hadfield, G., Magee, V. and Perry, C. B. 1934, The lysis of fibrin by streptococci. Lancet, **1**: 834–839.

32. Madison, R. R. 1934, Fibrinolytic streptococci from lower animals. Proc. Soc. Exper. Biol. & Med. **32**: 444–445.

33. Smith, F. R., Hankinson, C. L. and Mudge, C. S. 1936, Fibrinolytic activity of beta hemolytic streptococci from cow's milk. Proc. Soc. Exper. Biol. & Med. **34**: 266–270.

fibrin. Tillett,[34] in a review of this work, states that there is an apparent predilection of strains of hemolytic streptococci for the fibrin of a species homologous to that in which the organisms may survive, and, in some instances, invade. In experiments not reported in table 1, it was found that group C strain 1059, that caused an epidemic in guinea pigs, did not lyse guinea pig fibrin, although it lysed human fibrin. Group C strain 1083, isolated from an infected horse, failed to lyse either horse or human fibrin. Kaplan's[35] and later Christensen's[36] discovery that fibrinolysin depends upon two components, one of which is a proenzyme present as an inactive proteolytic factor in blood and the second an activating factor produced by streptococci, may explain the negative results obtained with guinea pig or mouse fibrin. Perhaps the activator produced by streptococci for the proenzyme in human blood is unable to activate a proenzyme in either guinea pig or mouse blood, or perhaps the proenzyme is not present. The fibrinolysin test as it is performed routinely employs human plasma.

Because of this specificity of action of fibrinolysin, the question might be raised regarding the influence of fibrinolysins derived from hemolytic streptococci of human origin on the course of infection in the mouse or the chick.

Tunnicliff[37] reported that the strongest lytic action was associated with virulent strains which formed capsules and produced smooth colonies. Dawson et al[15] stated that no significant differences in the fibrinolytic activity of M, S, and R variants of the same strain of hemolytic streptococcus were observed. Tillett[34] states that "findings indicate that although the production of fibrinolysin by hemolytic streptococci may frequently accompany the presence of experimental indices of pathogenicity (colonial structure and virulence for mice), the relationship is not an inseparable one."

The literature contains a large number of papers dealing with the fibrinolytic activity of strains of β hemolytic streptococci derived from various human infections. Tillett[34] reviewed these papers and stated that of 1299 strains tested by various workers, 899 (69%) were actively fibrinolytic. He also stated, however, that in the majority of reports the incidence of fibrinolytic activity by pathogenic strains was greater than 90%.

Because of the association of the various Lancefield groups with characteristic sources, some studies of the fibrinolytic activity of strains belonging to these groups have been made. Tillett[34] stated that of the 285 group A strains tested by various workers, 280 (98%) were actively fibrinolytic. There are a number of reports concerning the activity of strains belonging to groups B, D, E, F, and H. No fibrinolytic activity against human fibrin has been observed for strains belonging to any of these groups. Fifty-seven group C strains from normal persons or monkeys have been tested by various workers, and 54 of these were fibrinolytic. Seventy of 79 group G strains derived from normal individuals were fibrinolytic.

While the results reported in table 1 show no correlations between fibrino-

34. Tillett, W. S. 1938, The fibrinolytic activity of hemolytic streptococci. Bact. Rev. **2**: 161–216.
35. Kaplan, M. H. 1944, Nature and role of the lytic factor in hemolytic streptococcal fibrinolysis. Proc. Soc. Exper. Biol. & Med. **57**: 40–43.
36. Christensen, L. R. 1945, Streptococcal fibrinolysis: a proteolytic reaction due to a serum enzyme activated by streptococcal fibrinolysin. J. Gen. Physiol. **28**: 363–383.
37. Tunnicliff, R. 1936, Effect of dissociation of streptococci on their fibrinolytic and anticlotting activity. J. Infect. Dis. **58**: 92–97.

lysin production and virulence, Swift[20] suggests that "the presence of the proteolytic enzyme plasmin in a patient's blood might conceivably induce demonstrable lesions in his tissues; but no known pathogenic function has been definitely associated with streptokinase."

An inspection of table 1 and a perusal of the short discussion of results indicates that there is no correlation between the apparent generation time and the LD_{50} dose of streptococci for either the mouse or the chick. Studies on the rates of growth of hemolytic streptococci are not very numerous. Beckwith and Rose[38] reported that a strain of hemolytic streptococcus cultured routinely on artificial mediums grew much less rapidly than a strain of the same organism which was cultured over a period of five years by intrapleural passage in rabbits. The rabbit passage strain was quite virulent.

Mason[39] reported generation times of 26 to 37 minutes for hemolytic streptococci. Lewandowski and Stahly[8] studied the rate of growth of a group C hemolytic streptococcus, using the optical density technique. They found the growth to be very slow in neopeptone infusion broth and more rapid when this medium was enriched with horse plasma or glucose. Lewandowski and Stahly[8] later reported that the growth rates of five group A streptococci in plasma-glucose infusion broth varied from 54 to 69 minutes. The strain showing the highest virulence for mice had the slowest growth rate.

It is recognized that the optical density method does not necessarily indicate the actual number of organisms present in the culture at any given time, and it will be noted that the growth rate is expressed as apparent generation time. Henrici[40] points out that a variation in size of the cells occurs during growth. It is obvious that changes in cell size would affect the optical density of the culture, and Porter[41] cites experimental evidence to show that the nature of the medium, the temperature of incubation, and other factors affect growth during the logarithmic phase. In the experiments being reported, these factors were considered and controlled as far as possible.

Owing to the varying number of cells in chains of streptococci, the plate method was not employed. The determination of the optical density does give as true an indication of protoplasmic mass as it is possible to obtain, and it may be argued that the protoplasmic mass during the logarithmic phase of growth represents a true measure for growth.

Calculations based upon data given in table 1 give an average generation time of 36.4 minutes. This compares closely with that of 32, 34, and 35 to 37 minutes reported by Mason.[39] The range of 20 minutes to 82 minutes to be noted from an inspection of table 1 is greater than the range of 54 to 69 minutes reported by Lewandowski and Stahly.[8] The surprising observation is that the average generation time of 36.4 minutes should be in such close agreement with the findings of others, since it is well established that great

38. Beckwith, T. D. and Rose, R. J. 1925, Effect of medium on rate of multiplication, virulence and heat susceptibility of hemolytic streptococcus. J. Infect. Dis. **37**: 277–283.

39. Mason, M. M. 1935, A comparison of the maximal growth rates of various bacteria under optimal conditions. J. Bact. **29**: 103–109.

40. Henrici, A. T. 1938, Morphological Variation and the Rate and Growth of Bacteria, Springfield, Charles C Thomas.

41. Porter, J. R. 1946, Bacterial Chemistry and Physiology, New York, John Wiley and Sons, Inc., p. 131.

variation as to growth requirements among the Lancefield groups exists.

As previously stated, the results being reported in this paper show no correlation between apparent generation time in an enriched culture medium and virulence for either the mouse or the embryonic chick.

A product of some but not all strains of Lancefield groups A, C, and G that has been regarded by Todd[10] and others as related to virulence of streptococci is streptolysin O. Bernheimer and Cantoni[42] showed that streptolysin O had a cardiotoxic effect on the isolated frog heart. The literature bearing upon streptolysin O has been well reviewed by Todd,[10] Elliott[43] and Bernheimer and Cantoni.[42] While it is granted that streptolysin O may play a role in streptococcal infection, an inspection of table 1 shows no correlation with streptolysin O productions and the LD_{50} dose for either the mouse or the embryonic chick.

SUMMARY AND CONCLUSIONS

The preceding pages represent a study of 25 strains of β hemolytic streptococci belonging to Lancefield groups A, B, C, and D isolated from human and animal infections. The results may be summarized as follows:

1. All of 25 strains of streptococci pathogenic for man or certain animals were pathogenic for the chick. White Leghorn chicks were used in these studies. A comparison of the results with those obtained in a previous study using Austria White chicks indicates that the White Leghorn is, as a rule, more susceptible to streptococcal infections. Five of the 25 strains were avirulent for the mouse.

2. The mucous membrane of the mouse is more resistant to streptococcal infection than the chorioallantoic membrane of the chick. That the mucous membrane is not a perfect barrier in all cases is indicated by the fact that positive blood cultures were obtained quite frequently from apparently healthy survivors ten days after intranasal inoculation.

3. There is no correlation between the production of hyaluronidase in vitro and the skin spreading factor. Only 5 of 25 strains produced hyaluronidase in vitro, while 16 produced a definite spreading factor in the skin of rabbits.

4. The supernatants of 16 of the 25 strains produced inhibition or more than 50% of the phagocytosis of streptococci by mouse neutrophiles. The inhibition did not correlate with virulence for the mouse. The supernatants of 10 of the 24 strains inhibited by more than 50% the phagocytosis of streptococci by mouse monocytes. The inhibition did not correlate with virulence for the mouse. Phagocytosis of streptococci by mouse neutrophiles was intensified by the supernatants of one group C and two group D streptococci. These results did not correlate with virulence for the mouse. Intensification of phagocytosis by mouse monocytes occurred in the presence of supernatants of three group A, one group B, three group C and three group D streptococci. The results did not correlate with virulence for the mouse.

5. Only three strains (all group A) produce an oxygen-labile hemolysin, while 19 of 24 strains produce a leucocidin for mouse neutrophiles and only 11 of 23 produce a leucocidin for mouse mononuclear phagocytes. Only one of the supernatants of eight strains pro-

42. Bernheimer, A. W. and Cantoni, G. L. 1945, The cardiotoxic action of preparations containing the oxygen-labile hemolysin of *Streptococcus pyogenes*. J. Exper. Med. **81**: 295–306.

43. Elliott, S. D. 1945, A proteolytic enzyme produced by group A streptococci with special reference to its effect on the type specific M antigen. J. Exper. Med. **81**: 573–591.

duced marked inhibition of phagocytosis by human neutrophiles, and this strain did not produce an oxygen-labile hemolysin. Four of the seven remaining strains produced moderate inhibition and two no inhibition of phagocytosis. The supernatant of one of the strains producing an oxygen-labile hemolysin had no effect upon the phagocytosis of *Brucella abortus* by human neutrophiles.

6. The results show no correlation between the LD_{50} end point and such virulence factors individually or collectively as hyaluronidase, skin spreading factors, leucocidins, fibrinolysins, apparent generation time in vitro and streptolysin O. It would appear that while these virulence factors are important in establishing and disseminating infectious agents in the host and perhaps also in producing lesions, they are not either individually or collectively the lethal factors in infection.

7. The results suggest that some other factor or factors resulting from the interaction of parasite and host tissue is or are the lethal ones.

THE USE OF RADIOACTIVE PHOSPHORUS IN STUDIES OF VACCINIA INFECTIONS OF CHICK EMBRYOS

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Although there are at present a number of tests that can be performed for the detection of particular viruses in laboratory cultures, there still exists a need for an easily performed and rapid test which would be applicable to all viruses. In addition, such a test would be very valuable in those infectious diseases in which a virus is suspected as the etiological agent but not yet proved. Since phosphorus is associated with virus growth by virtue of its incorporation into nucleic acid, it was hoped that radioactive phosphorus (P^{32}) could be used to detect a virus or the effects of virus growth on the developing hen's egg.

Previous workers have succeeded in incorporating P^{32} into bacteria, plant viruses, bacteriophage, and into the encephalitis virus grown on the mouse brain, but the developing hen's egg has obvious advantages as a culture medium for viruses. Since the present investigation was begun, several other workers have reported on the results of using radioactive phosphorus to detect egg-grown viruses or the effects of virus growth on the eggs. Thus, Saenz and Taylor (1949) have studied the effect of influenza virus on the dispersion of P^{32} injected into the allantoic sac of embryonated eggs. Graham and McClelland (1950) have reported the incorporation of P^{32} into influenza virus grown on hens' eggs, using rather elaborate purification methods. Finally, Ward (1950) has shown that in hens' eggs infected with the common cold virus, the ratio of P^{32} found in the ribonucleic acid (RNA) fraction to that found in the acid soluble fraction of the allantoic fluid was from 2 to 30 times that for the control eggs. The viruses causing Newcastle disease and influenza were also studied by this method. Although Ward got positive results for the most part, he nevertheless calls attention to several experiments indicating failure of the method.

The above-mentioned concomitant work definitely demonstrates the feasibility of using radioactive phosphorus for the detection of egg-grown viruses. It was thought, however, that the results herein reported might still be of interest, since the method of purification (dialysis) employed in these experiments was simpler than the methods used by the other workers. The simplicity of the technique compared with chemical fractionation made possible the testing of material from individual eggs rather than pooled material. It therefore could reveal the wide variation in P^{32} activity from egg to egg, under similar treatment—a result not possible with pooled material. This feature of the work is unique in virus research in which comparable problems have been attacked.

MATERIALS AND METHODS

Vaccinia virus was inoculated onto the chorioallantoic membranes of developing hens' eggs into which P^{32} had been previously injected. The "window" method of inoculating the membranes was employed (Goodpasture, 1938; Burnet, 1942). The inoculum was prepared by

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taking the material from one "point" of vaccinia in calf lymph and diluting it to 1 ml in 0.85% saline. Four drops of the inoculum were placed on the membrane of each egg. The control eggs received 4 drops of saline.

The P^{32} was injected into the albumen of the egg. Other methods of getting the P^{32} into the eggs were first tried (feeding it to the laying hens and injecting it into the yolk), but these were found less feasible than the albumen route and were abandoned. P^{32} was usually injected into the egg not longer than one hour before the virus, exhaustive tests having first shown that a ½-hour interval was usually sufficient for the maximum transport of the P^{32} from the albumen to the allantoic membrane and other parts of the embryo. The amount injected into each egg was 0.5 μc in the earlier experiments, but in the later experiments 25 μc were used.

The virus was usually inoculated onto the egg on the 11th day of incubation, earlier inoculation usually resulting in high egg mortality. A series of preliminary experiments with P^{32} confirmed the chemical analysis of Kugler (1945) that before the 11th day of incubation there is very little phosphorus, either organic (nondialyzable) or inorganic (dialyzable) in the allantoic fluid, but that the amount rapidly increases after the 11th day, and that most of this increase is inorganic. In order to avoid the masking effect which this normal increase in phosphorus level might have on an increase caused by the virus, the eggs were usually not inoculated later than the 11th day after incubation. After various periods of virus growth, the allantoic fluid and chorioallantoic membrane of each egg were separately harvested and measured for P^{32} activity.

The allantoic fluid was aspirated into a tuberculin syringe, using a 20 or 22 gauge needle. Usually 1 ml was placed in a 3-ml metal counting cup for immediate evaporation under an infra-red lamp, and 1 or 2 ml of fluid were placed in a dialysis bag to be dialyzed for at least 12 hours against running tap water to remove inorganic P^{32}.

The entire chorioallantoic membranes or desired portions of them were excised, rinsed in sterile saline, weighed, and spun in the earlier experiments for 20- to 40-minute periods in a small-cup Waring blendor which was cooled by running tap water and to which 2 ml of saline were added for each membrane. This resulted in the breaking up of the cells and the release of virus particles when present. In the later experiments a heavy glass "homogenizer" attached to an electric motor was used for grinding up the membranes, 2 minutes usually being sufficient to complete the grinding of a single membrane with the homogenizer.

In most of the work, the allantoic membrane was cut into two portions, the one comprising the portion of the membrane beneath the artificial air space created by dropping the membrane (the "floor"), and the other the rest of the membrane, it having first been determined by long virus growth periods that pocklike lesions never developed beyond the margin of the membrane floor. Thus when virus growth periods too short for visible lesions to develop were employed, it was nevertheless possible to differentiate between the presumably infected and uninfected areas of the same embryo. These membrane portions are designated as the "inoculated" and "uninoculated" regions. Similar regions were harvested from control eggs that received no virus but which received all other experimental procedures. It was realized that the entire inoculated region was probably not infected, especially in the case of very short growth periods in the virus-inoculated group, but it was hoped that a sufficiently greater P^{32} uptake by the inoculated regions would be found to offset the presence of uninfected portions in these regions.

Each ground-up membrane was transferred to a 15-ml centrifuge tube and centrifuged at 2500 r.p.m. for 20 to 30 minutes to throw down the large particles of cellular debris. The supernatants presumably then contained the virus particles if they were originally present as well as other components of the cellular material too small to be sedimented by this method. The supernatants were then transferred to counting cups or dialysis bags or both, the total volume being measured in each instance, in order to convert the counts eventually to percent of total dose per gram of wet tissue.

Individual dialysis bags for the material harvested from each egg were made of Visking seamless cellulose tubing 36/32, 1¼ inch diameter inflated. The thickness of this tubing was 0.0026 inch. It was found that after 6 hours more than 97% of the total P^{32} was removed on the average from a sample, and that dialysis continued for as long as 72 hours increased this amount by less than 1%, a fact which indicated that there was little or no autolysis and subsequent release of inorganic phosphorus as a result of the more prolonged dialysis.

Radioactive phosphorus was obtained from the Oak Ridge National Laboratory in the form of very dilute carrier-free H_3PO_4, pH determinations showing a negligible amount of acidity. Suitable dilutions of the injection fluid used in each experiment were made and aliquots counted in order to arrive at the total dose of P^{32} injected into each egg.

EXPERIMENTAL RESULTS

P^{32} in allantoic fluids after 72 hours of virus growth

Vaccinia was inoculated onto the chorioallantoic membranes of 27 eggs (of 10 or 11 days' incubation) which had received 0.5 μc of P^{32} from 1 to 6 days previously and allowed to grow for 72 hours, at the end of which time all the membranes showed typical vaccinia lesions. (Smears were subjected to tests designed to rule out bacterial contamination; also, several successful vaccinia cultures were started from the infected membranes.) The undialyzed allantoic fluids from each of these eggs were measured for P^{32} content and their average P^{32} activity found not to be significantly different from that of 24 uninoculated eggs. Thus it was apparent that the virus particles or P^{32}-containing products associated with their growth were not being thrown into the allantoic fluid in sufficient quantities to be detected in the undialyzed allantoic fluids or were masked by the inorganic P^{32} present.

Dialyzed allantoic fluid (by contrast with the above undialyzed) was tested next (see table 1). The counts were made on samples of the allantoic fluids separately derived from individual eggs as mentioned under *Materials and Methods* (not on pooled material), except as otherwise noted in the table. It will be seen that the P^{32} activity of the inoculated eggs is significantly higher than that of the uninoculated eggs. These results are considered evidence that vaccinia virus can be detected in the allantoic fluid by the present method. As in the experiments using undialyzed allantoic fluid, all membranes of the virus-inoculated eggs showed typical vaccinia lesions in contrast to the absence of lesions in the control eggs.

TABLE 1.—*P^{32} activity of dialyzed allantoic fluid after 72 hours of virus growth (C/S/Ml).*

Inoculated eggs	Control eggs
2.0	1.1
1.6	0.5
1.6	0.1
4.9	1.2
7.6	0.4
1.4	1.0†
0.6	1.7‡
1.0	0.2
0.9	0.6
0.9	0.2
4.2	
0.4	
0.6	
3.6	
0.6	
0.9	
5.6	
2.9*	
Mean 2.3	0.7

Difference between means = 1.6 ± 0.7 C/S/Ml ($P = 5\%$).
C/S/Ml, counts/sec./milliliter.
* Pooled allantoic fluid from 4 eggs.
† Pooled allantoic fluid from 3 eggs.
‡ Pooled allantoic fluid from 2 eggs.

P^{32} in allantoic fluids after 1 to 30 hours of virus growth

Since there is an increased mortality associated with embryos inoculated earlier than the 10th day of incubation, it was hoped that sufficient virus growth would occur in 1 to 30 hours in eggs inoculated on the 10th day to allow the virus to be detected in the allantoic fluid before the normal rise in phosphorus level had taken place, thus eliminating the necessity for dialysis. However, the average P^{32} activity of both undialyzed and dialyzed allantoic fluids from two groups of inoculated eggs (one with virus growth of 1 to 12 hours and another from 12 to 30 hours) was not significantly different from the average P^{32} activity of their respective groups of uninoculated eggs.

P^{32} in chorioallantoic membranes

The fact that the presence of virus in the allantoic fluid could be detected after 72 hours of virus growth but not after the shorter periods used above may indicate that sufficient time must be allowed for the virus to invade the lower layers of the membrane before it can gain access to the allantoic fluid.

It is possible that at no time is a very large amount of virus thrown into the allantoic fluid and that most of it remains in the membrane. Therefore, it seemed desirable to see if P^{32} could be used to detect the presence of virus in the membrane itself.

In the first series of experiments on the membranes the virus was allowed to grow for 72 hours, the infected portions of the membranes excised, one egg served for both inoculated and control, the "floor" portion of the membrane being considered "inoculated" and the rest of the membrane as control, as outlined under *Materials and Methods*. It was found that there was still no significant average difference in nondialyzable P^{32} in supernatants from the inoculated and uninoculated regions of the membranes in a total of 18 eggs.

Since it had been noted that the uptake of P^{32} by the membranes was just as great in the shorter growth periods as in the longer, it was decided to shorten the growth periods still further. Accordingly, the P^{32} activities of dialyzed supernatants from the inoculated and uninoculated portions of the membranes of 62 eggs on which vaccinia virus had been allowed to grow for ½ to 4½ hours were compared. At each of the ½-, 1-, 1½-, 2-, 2½-, and 3-hour growth periods a significant difference was shown to exist between the averages of the supernatants of the inoculated and uninoculated regions of the same eggs, with the greatest difference occurring

TABLE 2.—*Effect of short periods of virus growth (½ to 4½ hours) on P^{32} activity of chorioallantoic membranes.*

Period of virus growth (hrs.)	Portions of membranes	P^{32} activity of dialyzed supernatants from allantoic membranes (C/S/G)*												Mean	Mean difference	Standard error
½	Inoc.	82	166	28	60	88								85	51	11.6
	Uninoc.	23	82	18	6	40								34		
1	Inoc.	70	75	155	560	41	112	22	13	23	12	62		104	89	43.7
	Uninoc.	15	10	23	37	10	16	14	10	15	11	6		15		
1½	Inoc.	200	750	1055	80	212	89	23	23	168				289	277	133.3
	Uninoc.	7	24	36	8	12	11	3	3	7				12		
2	Inoc.	37	28	51	75	126	165	46	460					123	98	48.2
	Uninoc.	10	5	34	60	24	24	6	32					25		
2½	Inoc.	50	290	96	70	110	44	57	209					116	98	38.5
	Uninoc.	8	24	56	25	12	7	12	6					18		
3	Inoc.	20	22	61	125	52	134	78	154					81	52	14.9
	Uninoc.	8	8	14	5	39	92	33	33					29		
3½	Inoc.	26	40	125	11	169								74	55	28.2
	Uninoc.	7	9	15	41	22								19		
4	Inoc.	25	38	330	44	80								104	84	52.6
	Uninoc.	13	8	36	14	27								20		
4½	Inoc.	190	52	60										103	86	54.0
	Uninoc.	22	6	22										17		

* P^{32} injection of 25 μc in 0.2 ml of saline per egg preceded inoculation of virus by 1 to 2 hours. C/S/G, counts/sec./gram.

weighed, macerated, and centrifuged. The amounts of P^{32} in the undialyzed and dialyzed supernatants were then compared with those of control eggs similarly treated except for the inoculation of virus. A large variation was noted from egg to egg, but no significant difference was observed between the average P^{32} uptake of the inoculated versus that of the control group.

The virus growth periods were next successively shortened by harvesting the membranes at 54, 48, 30, 27, 16, 15, and 12 hours after inoculation. In these experiments that involved periods of virus growth shorter than 72 hours,

at the 1½-hour period of virus growth (but with the difference of greatest significance occurring at the 3-hour period). Table 2 gives the data from these eggs.

To test the possibility that P^{32} uptake by the inoculated region of the membrane was greater than that of the uninoculated region because of injury incidental to dropping the membrane, exposure to air, or increased metabolism inherent in that region, and so not related to the presence of vaccinia virus, the following experiment was done. In 23 eggs into which P^{32} had previously been injected, openings in the shells were made and the chorioallantoic membranes dropped, with the formation of an artificial air space, just as is necessary preparatory to virus inoculation. However, no virus (or saline diluent) was inoculated, but the portions of the membranes underneath the air spaces and on which the virus would have grown (had the egg been inoculated) were cut out and their P^{32} activities compared with those of the portions unexposed to the air spaces. The average activity of these two portions was significantly different, that of the portions exposed to the air space being 40 C/S/G as compared with 14 C/S/G of the portions not exposed (and with an average of 20 C/S/G of the uninoculated portions of the 62 inoculated eggs shown in table 2).

However, when the difference between P^{32} activities of inoculated and uninoculated regions of membranes from all eggs on which vaccinia had been allowed to grow from 1 to 2 hours (table 2) were lumped together and compared with the corresponding difference in the P^{32} activities of the 23 controls (uninoculated), a highly significant difference was seen to exist, as was also the case when the growth period was extended from 1 to 2½ hours. But the difference failed to be significant when all eggs on which vaccinia had grown for ½ to 3 hours were lumped together and compared with the controls. The results of these experiments have been reported (Altenburg, 1950).

Although the difference between the inoculated and uninoculated groups of eggs was significant at the 99% level ($P = 1\%$) in the cases of the 1- to 2- and 1- to 2½-hour growth periods, it was decided to test the reproducibility of these results. It was also felt that the tests should include controls in which saline was dropped onto the lowered membranes (since saline had been used for the dilution of the virus), as well as controls on which only the operation of lowering the membrane was performed (as previously done). Since the greatest difference in P^{32} uptake by inoculated versus uninoculated regions had been shown to exist at the 1½-hour growth period, it was decided to use this as the growth period in subsequent experiments to test reproducibility. Because two sets of controls were run and the virus growth period was so short, not many eggs could be handled in one experiment.

In only one out of a total of 7 experiments (involving 110 eggs) was there a significant difference between the P^{32} activity of the inoculated and uninoculated regions either in the inoculated eggs themselves or in the controls. In one exceptional experiment, in fact, the uninoculated regions of individual eggs in a number of instances had a higher P^{32} uptake than the inoculated regions, in all three of the groups into which the experiment was divided: in the inoculated eggs, controls with saline, and controls without saline. Thus the later experiments, when individually considered, did not confirm the previous ones. However, the mean difference between the inoculated and uninocu-

lated regions of *all* the inoculated eggs (including the eggs shown in table 2 with 1½-hour growth period, making a total of 51 eggs) is 27.3 C/S/G, and that of the 81 control eggs (both with and without saline) is 10.9. The difference between these two means of 16.4 with its standard error of 7.2 is significant at the 96% level. When the one

take than the controls, after only 1½ hours of incubation, but a disappointingly large number of eggs must be used to establish this difference. It should be pointed out, however, that this fact is so apparent only because individual eggs, rather than pooled material, were tested.

Several eggs from each group in the

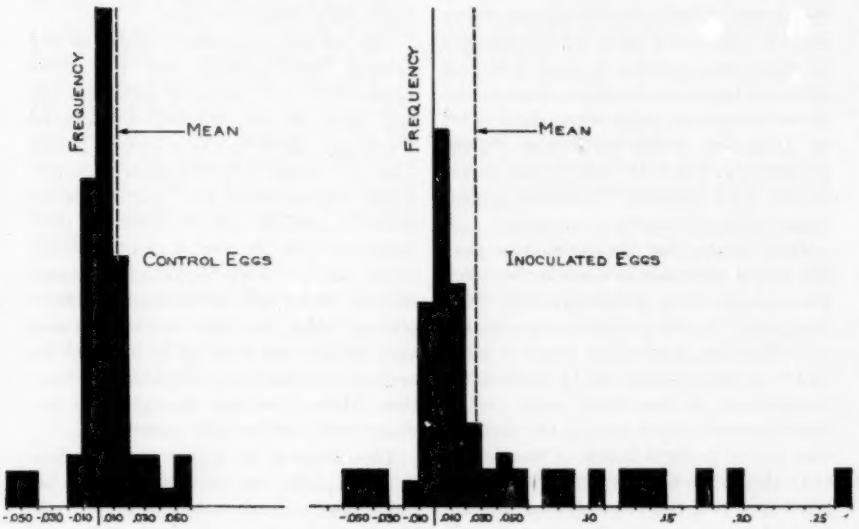

FIG. 1.—Histogram showing frequency of differences in P^{32} uptake between inoculated and uninoculated regions of each membrane from 51 eggs on which vaccinia virus had grown 1½ hours and 81 uninoculated eggs used as controls. (P^{32} uptake measured as counts/sec./gram, expressed as per cent of total dose; plus = inoculated greater; minus = uninoculated greater.)

very exceptional experiment mentioned above is omitted, the difference between the means of the two groups is 24.0 with a standard error of 9.9, which is significant at the 99% level. A histogram of these differences is presented (fig. 1). It is obvious that the histogram is spread out much more to the right in the case of the inoculated than in the control eggs, thus indicating an effect of the virus on phosphorus uptake.

Apparently, then, vaccinia-infected membranes do show a greater P^{32} up-

exceptional experiment mentioned above were assayed for both nondialyzable and dialyzable P^{32} content, in order to see if any correlation could be made between the P^{32} distribution throughout the egg and the variation of the P^{32} in the membranes from egg to egg. It was found that high P^{32} activity in the undialyzed embryos could be correlated with high levels of P^{32} found in the dialyzed membranes. It might therefore have been preferable to make all comparisons between inoculated and

uninoculated regions of the membranes on the basis of P^{32} activity of the undialyzed embryos from each egg rather than on the total amount of P^{32} injected into each egg.

Infectivity tests were reasonbly successful. It was not feasible to test all the inoculated eggs at the 1½-hour growth period for the presence of virus, but 6 out of 9 membranes tested by dropping fragments of the infected regions under aseptic conditions onto the membranes of other eggs produced typical lesions after 72 hours' incubation. Two of the three remaining eggs were dead. Out of 10 eggs on which portions of the supernatants from 10 inoculated membranes were dropped, 7 showed typical lesions after 72 hours' incubation.

In order to test the possibility that the strain of vaccinia used in the later experiments was slower-growing than that used in the previous experiment, and therefore might not reach a peak of P^{32} uptake as early as 1½ hours after inoculation, 6 inoculated eggs and 5 saline controls were tested, the incubation period being 4 hours. It was found that the difference between the inoculated eggs and the controls was not increased by increasing the virus growth period.

DISCUSSION

The use of allantoic fluid and of chorioallantoic membranes for the detection of vaccinia virus may now be contrasted. Possibly because little of the virus normally is thrown into the allantoic fluid, a long growth period (72 hours) is necessary to demonstrate its presence in the dialyzed fluid. In the dialyzed supernatants from the membranes, however, an extremely short growth period (1 to 2½ hours) is sufficient, and in fact, necessary, to demonstrate the presence of virus. Longer periods allow for the replacement of radioactive phosphorus by nonradioactive (phosphorus turnover), thus obliterating any initial difference in P^{32} uptake between inoculated and uninoculated regions. That much of the P^{32} being measured in the undialyzable portions of the inoculated membrane supernatants or in the allantoic fluid is actually combined in the virus particles is open to question. However, for simple detection of the presence of virus, this is unimportant.

One of the surprising results of the present investigation was the great variation in P^{32} uptake from egg to egg, both by the infected membranes themselves and by the allantoic fluids. This fact would not have been apparent if the measurement had been made on pooled material. But dialysis, in conjunction with the use of radioactivity, made possible work on individual eggs, since even though the amounts of phosphorus looked for from single eggs was very small—too small to be handled by ordinary methods of chemical purification—their detection through their radioactivity was readily possible.

The causes of the egg to egg variation in P^{32} uptake are undoubtedly to be found partly in the technique employed. However, the greatest single factor in the variation probably lies in the eggs themselves. Buddingh (1950) says: "Under the proper conditions of incubation the fertile egg proceeds at a furious rate. From hour to hour, day to day, the successive steps of development and differentiation which complete the hatched chick within 21 days confront us with a series of biochemical and biophysical situations to which viruses are variously adapted."

A second surprising fact resulting from the present work is the extreme rapidity with which P^{32} injected into the albumen makes its way into the chorioallantoic membrance, becomes incorporated as undialyzable phosphate,

and is released into the allantoic fluid. This has been amply proved by studies on at least 200 individual eggs.

SUMMARY

By means of radioactive phosphorus (P^{32}) it was possible to demonstrate virus growth in developing hens' eggs, both in the infected chorioallantoic membranes and in the allantoic fluids. The testing of individual eggs was made readily possible by the use of dialysis as a method of purification, and it revealed an extraordinary variation in P^{32} uptake from egg to egg. This very fact, however, made necessary the use of a large number of eggs to detect a significant difference in P^{32} uptake by eggs inoculated with the virus and the controls.

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SPECIFIC, COMPLEMENT-DEPENDENT HEMOLYSIS OF SHEEP ERYTHROCYTES BY ANTISERUM TO AZO HAPTEN GROUPS

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Recently it has been observed[1,2] that red cells exposed to various bacterial polysaccharides can be hemolyzed in the presence of complement (C′) by antiserum against the corresponding bacteria. This is in accord with the classical concept which assumes that specific hemolysis of red cells depends primarily on the fixation of C′ at the surface of the cell rather than on a specific combination of antibody with some particular component of the red cell.[3,4] It is known that antiserum to simple haptens will form specific, stable combinations with sheep stromata or red cells bearing the corresponding hapten. Thus Landsteiner and Van der Scheer[5] used hapten-azo-stromata for absorption of individual components of antiserums to mixed antigen injections, and Pressman et al[6] found that hapten-azo-red cells were specifically agglutinated by antiserum to the hapten group. This work strongly suggests that red cells coupled with a simple azo compound might be hemolysed by C′ in the presence of antiserum to this hapten group. If this hemolysis occurred at low serum concentrations and was quantitatively dependent on the amount of antibody present, it would provide a valuable method for quantitative estimation of antibodies against labeled proteins. It would also provide a convenient quantitative method for investigating serum specificity and cross reactions to various haptens. Furthermore, such a hemolytic system would be of considerable theoretical interest in investigating the nature of complement action.

The present paper reports observations establishing the specific hemolysis of sulfanilic acid-azo-sheep erythrocytes (SA cells) by rabbit anti-SA-bovine-γ globulin (SAgG) and anti-SA-ovalbumin (SAOV) in the presence of fresh guinea pig serum.* This reaction was found to be specific with respect to the hapten-azo group and to resemble the ordinary hemolysis of red cells by anti-red cell hemolysin with respect to magnesium activation, citrate sensitivity, and complement requirement and to follow the usual von Krogh relationship for immune hemolysis. This general phenom-

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* While this paper was in preparation, Silverstein and Maltener reported the specific hemolysis of sheep cells coupled with diazo-arsanilic acid by antiserum against the arsanilic acid group at the meeting of the Federation of American Societies for Experimental Biology.[7]

7. Silverstein, A. M. and Maltener, F. 1952, Fed. Proc. **11**: 481.

enon of hemolysis of erythrocytes by antiserum specific for a coupled hapten group (or protein molecule) but having no affinity for normal cells will be referred to as haptenic hemolysis.

MATERIALS AND METHODS

Buffers.—Buffer #1 was prepared from 50 ml stock sodium chloride containing 17 g/100 ml, 50 ml 0.1 *M* sodium phosphate adjusted to give pH 7.3 to 7.4 in final salt solution, 500 ml water and 10 g c.p. dextrose, all made up to 1 liter with water.

Buffer #2 was prepared from 10.2 g sodium-5,5-diethyl barbiturate, 500 ml stock sodium chloride as above, 1 liter of water, 10 ml of a solution containing 3.8 ml 10 *M* hydrochloric acid (exact amount adjusted to give final pH of 7.3 to 7.4 with a given lot of barbiturate), 0.023 g $CaCl_2 \cdot 2H_2O$ and 1.0 g $MgCl_2 \cdot 6H_2O$, all made up to 2 liters with water. The barbiturate was completely dissolved before adding acid. This is similar to the buffer recommended by Mayer et al,[8] except that they include bicarbonate and use 0.2 g $CaCl_2 \cdot 2H_2O$.

For use a fresh dilution of 200 ml of the above plus 10 ml of 20% bovine serum albumin (BSA) and 10 g c.p. dextrose up to 1 liter with water was prepared every 2 to 3 days. The BSA was Fraction V powder, Armour & Company, Chicago, and the 20% solution was stored frozen in 10-ml portions.

Buffer #3 was similar to #2 but contained only 5 ml of 20% BSA per liter.

Buffer #4 was similar to #3 but contained no dextrose.

Insofar as possible all buffers were kept refrigerated at all times.

Preparation of sulfanilic acid-azo-cells (SA cells).—Sheep blood was drawn aseptically into an equal volume of sterile Alsever's[9] solution and the cells were used within 24 hours. All glassware used to handle the cells was first soaked overnight in dichromate-sulfuric acid cleaning solution. One volume of packed cells (10 minutes at 600G calculated for the bottom of the tube) was washed 4 times with 10 to 20 volumes of buffer #1. The following diazo solution was added, with stirring during about one minute, to 12 ml of a 50% suspension of the washed cells in buffer #1 at 0 to 3 C: 1.8 ml 0.029 *M* sulfanilic acid (5 mg SA/ml), 0.9 ml 0.2 *M* hydrochloric acid, and 1.8 ml 0.05 *M* sodium nitrite. After standing for 10 minutes at room temperature 0.6 ml 0.1 *M* urea was added. Again after 10 minutes at room temperature and chilling 0 to 3 C, 5.4 ml buffer #1, 0.9 ml 0.5 *M* sodium phosphate pH 7.65, and 0.6 ml 0.2 *M* sodium carbonate were added.

Final pH of the diazo solution was 7.5 to 7.7, and it was added to the cells immediately after neutralization. After a suitable time for coupling (10 to 60 minutes for practical purposes), the suspension was poured into 10 to 20 volumes of cold buffer #2. The cells were centrifuged and washed twice in the cold with buffer #3 and finally made up to a 1% suspension in buffer #2. This suspension was standardized photometrically so that 1 ml plus 4 ml water read 150 ± 5 in the Klett-Summerson colorimeter, using a 540 filter and standard Klett tubes. For normal cells and cells coupled up to 30 minutes with diazo-SA, this gave a suspension containing $1.95 \pm 0.1 \times 10^8$ per ml.

Some experiments with radioactive SA indicate that under the above conditions 2 minutes of coupling gave 7×10^4 SA groups/cell; 10 minutes, 3×10^5; 60 minutes, 1×10^6. The cells coupled for 60 minutes formed a stable combination with only 0.6% of the total SA used in the preparation; and 60% could still be found as free diazo-compound by a colorimetric determination on the supernatant.

The susceptibility of the cells to spontaneous hemolysis was a function of the time of coupling. Cells coupled 10 minutes or less were practically as stable as normal. When cells stored as a 1% suspension in buffer #2 were used for hemolysin titrations, 10-minute cells gave less than 6% hemolysis in controls after 5 to 7 days storage, 30-minute cells gave 10% hemolysis after 3 days, as did 60-minute cells after less than 12 hours. The hemolysis of 3-day-old 30-minute cells could be reduced to less than 5% by centrifuging and resuspending in fresh buffer #2. If the BSA is omitted from the buffer, 10-minute cells are 10% hemolyzed in about one day. Gelatin extends the life somewhat but is much less efficient than BSA. Cells could be stored for longer periods if they were returned to the Alsever's solution plus plasma from which they were originally obtained. After 5 weeks of such storage with subsequent washing in buffer #2, one batch of 10-minute cells gave less than 5% hemolysis in controls during the first day and 30-minute cells gave 10 to 12%. Batches of coupled cells were prepared from 5 different sheep. Although some cells may have been less fragile than others, no striking or consistent differences were observed.

Preparation of arsanilic acid-azo-cells (AA

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cells).—The procedure was similar to that for SA cells except 2.2 ml of 0.5% arsanilic acid was substituted for the SA, the solution was chilled to 0 to 3 C before adding nitrite, and diazotization was allowed to proceed for 30 minutes. The time of coupling was as long as four hours, but cells coupled more than two hours were too unstable to be of much use. Two hour AA cells were about as stable as one hour SA cells.

Preparation of protein-azo-benzidine-azo-cells.—The initial coupling with benzidine was similar to that with AA, using 9 mg of benzidine dissolved in 2 ml water after adding the hydrochloric acid. After 10 minutes or 30 minutes in the benzidine solution, samples of the cell suspensions were mixed with an equal value of 1% ovalbumin (OV) or SAgG for 30 minutes and then with 10 to 20 volumes of buffer #2 and the preparation completed as for SA cells.

These cells gave 10 to 15% hemolysis in controls for titrations started within 2 hours after completing the preparation, and about 25% hemolysis in controls after 12 to 18 hours.

Hemolysin titrations.—Hemolytic activity was determined essentially as described by Taliaferro and Taliaferro,[10] using the 50% end point measured photometrically. Cells were suspended in buffer #2 and all other dilutions were made with buffer #4. The cells were incubated with serum for 10 minutes at 37 C and then chilled to 0 to 3 C before adding C′. At the end of the incubation with C′ the tubes were again chilled to 0 to 3 C and 0.1 ml of molar sodium citrate was added to stop the reaction before centrifuging. A control with serum omitted and one with complement omitted from the complete system were run on each titration, and the Klett reading of the more highly hemolyzed control was subtracted from the readings of the other tubes. Insofar as possible, titrations were performed with cells showing less than 10% hemolysis in the controls, but in all cases a Klett reading of 150 above the control was taken as the 50% end point.

Complement used for most of the titrations was absorbed with 1/20 of its volume of sheep cell stromata for 10 minutes at room temperature plus overnight at 0 to 3 C. The stromata were centrifuged off and the C′ stored in 2 ml portions in stoppered plastic tubes at −50 C. Lyophilized C′ was obtained from Carworth Farms Inc., New City, New York.

Agglutination titrations.—One ml of 0.5% cell suspension was mixed with 0.5 ml of doubling dilutions of serum and incubated at 37 C for 1 hour. The tubes were then chilled, centrifuged at 600 G for 10 minutes and read by observing the behavior of the cells on resuspension. The titer is reported as the number of the tube containing the highest dilution of serum in which cells showed a distinct tendency to suspend in clumps when compared to a control of the same serum with normal cells. The coupled cells were not agglutinated by normal serum. To obtain the actual final dilution of serum in 1.5 ml, take 2 to the power indicated by the tube number and multiply by 15.

Quantitative precipitin determinations.—Precipitating antibody specific for the hapten group was determined as described by Kabat and Mayer.[11] The serums were incubated at 37 C for 1 hour and then 9 to 10 days at 0 to 3 C. Controls of each serum plus saline and each antigen plus normal serum were all negative after 10 days. Total nitrogen in the precipitates was determined by Kjeldahl digestion and Nesslerization as described by Groman.[12] These serums did not give a sharp equivalence zone with inhibition by excess antigen in this heterologous precipitation. The serums were precipitated, usually in duplicate, with about 0.7, 1.0, and 1.4 times the amount of antigen corresponding to the Dean-Webb optimal proportion ratio. Although even the smallest amount of antigen left an excess in the supernatant, the largest amount usually gave as much or more precipitate than any other. Three serums which were set up with ½, 1, and 2 times the optimal proportion amount of antigen gave progressively greater amounts of precipitate with increasing antigen after 48 hours, even though for one the smallest amount was in excess. However, for most of the serums the difference between the two largest amounts of precipitate was only 2 to 5% of their average, the largest difference being 15%. Therefore, the largest amount of precipitated nitrogen is considered to be a reasonable estimate of the relative amounts of precipitable antibody specific for the hapten group.

The antigens had been prepared several years prior to their present use. The SAgG and SAOV were prepared as described previously[13] and the AAgG was kindly donated by Dr. J. W. Moulder. All were repurified shortly before use by two reprecipitations and dialysis against three changes of phosphate saline as described in an earlier paper.[13]

10. Taliaferro, W. H. and Taliaferro, L. G. 1950, J. Infect. Dis. **87**: 37–62.
11. Kabat, E. A. and Mayer, M. M. 1948, Experimental Immunochemistry, Springfield, Charles C Thomas. pp. 59–62.
12. Groman, N. B. 1951, J. Infect. Dis. **88**: 126–150.
13. Ingraham, J. S. 1951, J. Infect. Dis. **89**: 109–116.

Serums.—None of the serums used was prepared expressly for this work. They were of diverse history, most having been used for instruction purposes. Many showed heavy bacterial contamination and all were clarified by 30 minutes centrifugation at 10,000 r.p.m. in a refrigerated Servall SS-1 centrifuge and preserved with merthiolate in 1:10,000 final concentration.

All except the anti-stroma #11 were absorbed with 1/10 volume of sheep stromata for 30 minutes at 37 C and overnight at 0 to 3 C, and had less than two 50% units per ml of anti-normal sheep cell hemolysin.

TABLE 1.—*Effect of intensity of coupling on hemolytic titer.*

Cells	50% units of hemolysin/ml serum	
	Anti-stroma #11	Anti-SAgG #3A
Normal	1200–1450 (3)†	<2
1/100 2-minute SA	1060	<2
1/10 2-minute SA	1200	<10
2-minute SA	1060	125
5-minute SA		210*
10-minute SA	1200–1450 (6)	750
30-minute SA	1400	2000

Excess diazo compound could be detected in the supernatant of the cells coupled in 1/10 the regular concentration of reagent but not in 1/100 by a colorimetric test sensitive to about 1 μg of SA/ml.

* Serum 3B.

† Figure in parenthesis indicates number of determinations. Several different lots of cells were used.

EXPERIMENTAL RESULTS

The data of tables 1 and 2 show the magnitude of the titers obtained in this haptenic system and give an indication of the quantitative significance of the results. It is apparent from table 1 that the titer depends very much on the intensity of coupling of the cells. Extensive coupling did not make the cells any less sensitive to immune hemolysis or alter in any way so far detected their reaction in the normal hemolytic system. The 30-minute cells of table 1 have about 6×10^8 SA molecules per cell but still give the same titer as normal cells with anti-stroma serum.

Columns 1 and 2 of table 2 show that even when coupled for the same length of time, two preparations may give

TABLE 2.—*Anti-hapten activity of various serums.*

Serum*		Hemolysin 50% units per ml			Agglutinin‡		Anti-hapten precipitin§ μg N_3/ml
		30-minute SA-cells			30-minute SA cells	2-hour AA cells	
		6/11	6/24	Ratio 2:1			
		1	2	3	4	5	6
Anti-SAOV	8	155	70	.45	7– 9		28
	9	4.5	<1		5– 9		32.5
	12	†			10		58
	15	230	108	.47	9–10		26
	106	420	310	.74	9	(>5)	174
Anti-SAgG	2A	195	140	.72	(7)		0
	2B	1200	940	.78	10	(>5)	15.5
	2C	94	67	.71	(6)		0
	3A	1950	1450	.74	10		83
	3B	1200	940	.78	8–10		65
	4A	<1	<1		(5)		0
Anti-SAgG mice	7/13	<1			(3)		0
	12/ 6	2			(8)		0
		2-hour AA cells					
		6/23	6/30				
Anti-AAOV	40		30			(7)	40.5
	41	18	30	.60	(4)	(8)	(63)
	42		12.5			(6)	(33.5)
	43	8	14	.57	(2)	(8)	25.5

Agglutinin and precipitin determinations given in parentheses are from single determinations. Others are from duplicates.

* Serums are numbered for rabbits in which they were prepared. Letters after a number refer to separate bleedings from the same rabbit.

† Twelve gave 30% hemolysis at dilutions from 1:2 to 1:10 and then less at higher dilutions.

‡ Reported as number of last tube showing agglutination. See *Materials and Methods.*

§ Anti-SAOV serums precipitated with SAgG, anti-SAgG with SAOV, and anti-AAOV with AAgG.

TABLE 3.—*Haptenic hemolysis with protein-a*o-benzidine-azo-cells.*

Serum		50% hemolytic units/ml serum				
		10-minute OV cells	30-minute OV cells	10-minute SAgG cells	30-minute SAgG cells	30-minute SA cells
Anti-gG	19	0	0	45	30	0
	122					0
Anti-OV	21	230	56	0	0	
Anti-SAOV	106	10		210	200	400*
Anti-SagG	3B	0		320	220	1000*

* Average values.

very different titers. However, for six of eight anti-SA serums, the ratios of titers with the two preparations were constant within ±5%. The difference between the two preparations of table 2 may be about the maximum to be expected since two other lots of 30-minute cells gave titers of 900 and 1100 with serum 3B. The maximum deviation between duplicate titrations of serums 8, 106, 2C and 3B performed on different days with the same lot of cells was 3%. The variability of titrations with 10-minute cells was about three times as great. The agglutination titers given in columns 4 and 5 of table 2 are very roughly proportional to the hemolytic titers. These agglutination titrations are apparently a more sensitive test for antibody than the hemolytic titrations, which is the reverse of the situation with anti-normal sheep cell serums. The agglutination tests showed a very definite cross reaction between anti-SA and anti-AA serums. This cross reaction was barely detectable with the hemolytic system, serum 3B having 9 units/ml of anti AA-cell hemolysin.

With the exception of serums 9 and 12 there is an approximate correspondence between anti-hapten precipitin content and hemolytic activity. Serums 9, 12, 15, 106, 2B, and 3B were tested and all found to be considerably more anticomplementary than a normal serum which had been stored frozen since it was drawn. Serums 9 and 12 had by far the highest anticomplementary activity. Since the haptenic system as we have it set up was especially sensitive to this anticomplementary action this might account for the discrepancies between hemolytic activity and precipitin content. However, the quantitative reliability of the reaction was not of primary concern in this phase of the work and it will require further work with fresh serums to determine whether the hemolytic titer can serve as a reliable estimate of precipitin content.

The data of table 3 are only very roughly quantitative, most of them being estimates from the hemolysis of two dilutions of serum differing by tenfold. However, they indicate the specificity and possible scope of "haptenic hemolysis".

The specific inhibition tests reported in table 4 are further evidence that the specificity of this reaction is similar to that of the precipitin reaction. The inhibition test was performed by incubating the serum with an equal volume of the indicated substance for 15 minutes at 37 C and then setting up dilutions of this serum-inhibitor mixture against the cells. Hemolysis by SAgG was completely inhibited by an equal volume of 0.01 *M* SA-tyrosine when serum plus inhibitor was diluted from 1:5 to 1:80, but as the mixture was further diluted, some hemolysis

TABLE 4.—*Specific inhibition of haptenic hemolysis.*

Antiserum and cells	50% units of hemolysin/ml serum Serum incubated with equal volume of:		
	Veronal buffer	SA-tyrosine 0.01*M*	AA-tyrosine 0.01*M*
Stromata #11 Normal cells	1200	1260	1260
SAgG #3 10-minute SA cells	750	< 2	840
AAOV #42 60-minute AA cells	53	63	< 5

TABLE 5.—*Activity of C′ in normal and haptenic systems.*

Serum	Anti-stroma #11 or anti-cells #301A	Anti-SAgG 3B		
Cells	Normal	10-minute SA	30-minute SA	60-minute SA
Relative C′ titer*				
Mean	1	0.54 (4)	0.89 (3)	1.09 (1)
Range		0.44–0.63	0.84–0.94	
Slopes of Von Krogh plots				
Mean	.26 (6)	0.25 (6)	0.26 (5)	0.25 (1)
Range	.22–.32	0.20–0.30	0.21–0.32	
Titer of pooled guinea pig serum stored at −70 C	160	130		
Titer of above after 10 days at 0 C in 1:100 dilution	42	38		

Figure in parenthesis indicates number of determinations.
* Lyophylized C′.

amounting to about 20% occurred at 1:330.

The available evidence indicates that the action of C′ in this haptenic system is strictly analogous to that in normal hemolysis. The data of table 5 show that the titer of C′ in this system approaches that of the normal hemolytic system as the extent of coupling is increased. The loss of C′ activity from fresh guinea pig serum on storage is similar for the two systems, and serum heated to 56 C for 20 minutes in 1:50 dilution was completely inactive in both. There is no difference in the slopes of the von Krogh plots. Data of table 6 show that the enhancement of C′ activity by magnesium and inhibition by citrate are at least qualitatively similar in the two systems. Typical plots of the data from C′ and hemolysin determinations are shown in figure 1. The data from hemolysin titrations in the haptenic system usually gave good straight-line Von Krogh plots with slopes between 0.4 and 0.5. The mean slope from nine titrations of anti-stroma #11 was 0.39 with a range from 0.34 to 0.48. Serums having low titers (less than 100) and cells coupled less than 10 minutes sometimes gave steeper slopes or curves which were concave upward. Such upward concavity might result from intense anticomplementary activity at high concentrations.

DISCUSSION

The observations reported here lead to the conclusion that coupling sheep erythrocytes with a hapten group such as SA simply confers on them a new specificity without masking the old and that hemolysis by C′ in the presence of antiserum to the attached group is then strictly analogous to ordinary immune hemolysis of normal cells. This does not seem unreasonable when we consider that the surface area of the red cell is

TABLE 6.—*Effect of magnesium, calcium and citrate on C′ titer in normal and haptenic system.*

	Klett-Summerson reading minus control	
	Anti-stroma #11 with normal cells	Anti-SAgG #3A with SA cells
Mg+Ca as fraction of 60+3μg/5 ml	C′ 1/100	10-minute cells C′ 1/100
None	14	8
0.01	56	21
0.1	169	69
1	246	157
Sodium citrate molarity	C′ 1/250	2-minute cells C′ 1/75
None	140	190
0.00001	121	182
0.0001	108	177
0.001	13	92
0.01	0	0

over 10^{10} Å^2. If we assume that the aromatic residues in the surface of the cell are preferentially bisubstituted and that every two SA groups effectively block an area 10 Å in radius, the 6×10^5 SA groups coupled to 30-minute cells would block slightly less than 1% of the surface of the cell. However, consideration of the size and the number of protein molecules available for coupling in the preparation of the protein-benzidine cells shows that the situation here might be different. If the SAgG molecule were attached in a manner to block the largest amount of cell surface (7×10^3 Å^2 for an ellipse 220×40 Å) and if about 5% of the available protein molecules were taken up instead of about 0.3% as for SA, there would be 4×10^5 SAgG/cell and about 25% of the surface would be covered. Therefore, it seems possible that the lower titers for the more intensely coupled cells of table 3 might be owing to protection of the cell from C′ by the OV or SAgG plus attached antibody.

There seems to be no obvious explanation for the low titers in the arsanilic acid system unless possibly the serums all happen to be similar to 9 and 12. No direct measure of extent of coupling was attempted, but the two-hour AA cells showed a somewhat greater color change and increase in fragility than 30-minute SA cells, and it seems reasonable to assume that they coupled at least as much hapten as did 10-minute SA cells. A colorimetric test showed a large excess of diazo-AA in the supernatant at the end of 2 hours.

It seems likely that the hemolytic titer of a serum against cells coupled with SA and possibly with any hapten or even protein antigens may provide a reasonably good estimate of its content of precipitable antibody against the coupled group. In any case, these hapten hemolytic titers are a measure of a specific activity of serum and appear to be reproducible with about the same precision as ordinary hemolytic titers. Therefore this phenomenon may serve

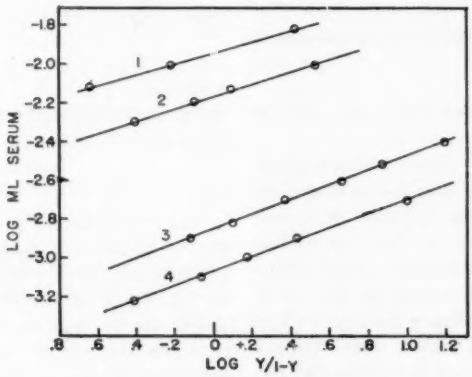

FIG. 1.—Von Krogh plots of data from C′ and hemolysin titrations. (1) Titration of "Lyovac" C′ with anti-SAgG 3A and 10-minute SA cells. (2) "Lyovac" C′ with anti-stroma #11 and normal cells. (3) Titration of anti-SAgG 3A with 10-minute SA cells. (4) Titration of anti-stroma #11 with normal cells.

as an independent measure of antibody formation and may make it possible to determine a large variety of antibodies using the simplicity and precision of the hemolysin titration.

The agglutination reaction is of even greater sensitivity and has made it possible to demonstrate anti-hapten specificity toward SA in a number of serums which gave no reaction in the usual heterologous precipitation tests or even by haptenic hemolysis. It is of special interest that a definite cross reaction between anti-Sa and anti-AA serums have been observed since, on the basis of precipitin tests, Landsteiner[14] reported that this reaction does not occur.

Finally, this system is of interest as a possible means for investigating the mechanism of C′ action. By using haptenic hemolysis it will be possible to study the hemolytic action of C′ on cells sensitized by an antibody which is as homogenous as possible and of a type carefully studied by quantitative immunochemical methods. In addition, the number and nature of the antigenic groups on the cell can be known and under the control of the investigator. Since the antibodies used in the present investigation appear to play the same role in immune hemolysis as classical hemolysins, it seems that any possible enzymic role which may be assigned to the latter[15] should also apply to these antibodies. The apparent difference in sensitizing efficiency of anti-SA and anti-AA may be of particular interest with respect to this question.

14. Landsteiner, K. 1947, The Specificity of Serological Reactions, revised ed., Cambridge, Harvard Univ. Press.

15. Bowman, W. M., Mayer, M. M. and Rapp, H. J. 1951, J. Exper. Med. **94**: 87–110.

SUMMARY

It has been found that sheep erythrocytes coupled with sulfanilic acid are hemolyzed by C′ in high titer in the presence of antiserum specific for the SA group but having no affinity for the normal cells. This hemolysis has the same quantitative dependence on serum and C′ concentration as ordinary hemolysis. Complement appears to act just as it does in normal hemolysis and shows similar magnesium requirement and citrate sensitivity. The specificity of the reaction is similar to that of the ordinary precipitin reaction but the sensitivity is much greater. When the coupled cells are used in agglutination tests the sensitivity is still further enhanced. This reaction has been found to occur also with cells coupled with arsanilic acid and through bis-diazotized benzidine to ovalbumin and sulfanilic acid globulin and is apparently a general phenomenon.

COMPARISON OF THE TROPISMS OF SIX STRAINS OF NEWCASTLE DISEASE VIRUS IN CHICKENS FOLLOWING AEROSOL INFECTION

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The isolation of Newcastle disease virus from the air of a poultry house,[1] the spread of the infection to chickens separated from diseased birds except for air they breathed, and the signs of coughing and rales quite common to the disease suggest that the respiratory tract is an important route of infection in chickens. Entrance of infection occurs by other channels since the chicken has been found to be susceptible by various routes of inoculation.[2,3] Chickens inoculated intramuscularly, intravenously or intranasally develop Newcastle disease, yet the course of the infection and sometimes the outcome is not the same as in chickens acquiring the disease by natural exposure to the virus.[2–5]

The simulation of natural infection by placing susceptible birds in cages with infected ones has several experimental disadvantages. As yet, too little is known about the quantity of virus that an infected bird expels or the duration of virus excretion. In contact exposure the infection of one individual obviously is dependent, in part, upon the stage of infection in another individual. The vagaries of environment and circumstance which admittedly are operable in an epizootic make it impossible to evaluate quantitatively or to determine accurately the progression of events in the course of the disease. Recently we have adapted a simple nebulizing device for the exposure of individual chickens to aerosols. The instrument makes possible comparative titrations between strains of Newcastle disease virus that are reproducible with reasonable accuracy.

An initial study has been concerned with the quantitative aspects of pathogenicity of six strains of Newcastle disease virus for chickens exposed to aerosols of the virus. Observations have been directed to that period—the fourth day after exposure—when signs of disease usually become prominent.

MATERIALS AND METHODS

The virus strains were obtained from the repository maintained at the University of Wisconsin, with the cooperation of the Bureau of Animal Industry and the North Central Regional Technical Committee on Newcastle disease.[6] The

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6. North Central Regional Technical Committee on Newcastle Disease, University of Wisconsin mimeographed publication, November, 1950.

strains were selected to represent extremes of pathogenicity.

R0, a strain of a moderate degree of lethality for chickens, was isolated by J. R. Beach at the University of California in 1944. CG 179, also lethal for chickens, was isolated two years later, 1946, in the same laboratory. Strain GB was recovered by W. A. Boney from a severe outbreak of Newcastle disease in Texas in 1948. It kills a large proportion of chickens exposed to it.

Strain KM, or Manhattan, was isolated by L. D. Bushnell at Kansas State College in 1948. The strain possesses a low pathogenicity for chickens. Strain Roakin was isolated in New Jersey in 1946 by F. R. Beaudette. It possesses a very low pathogenicity for chickens and is widely used as a living vaccine by the wing-web method. B1 was isolated by S. B. Hitchner from a culture originally designated as infectious bronchitis. Because of the very low pathogenicity for chickens it has been used as a living vaccine which is introduced intranasally or intraconjunctivally.

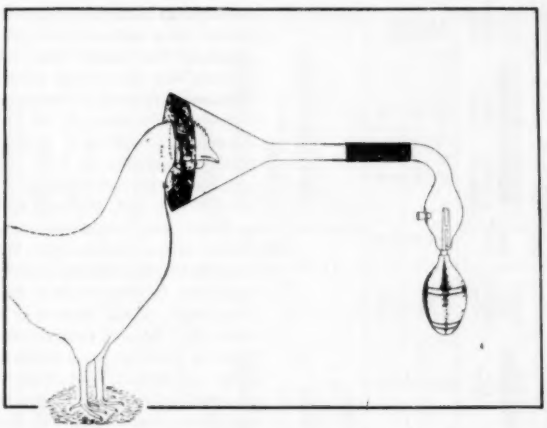

FIG. 1.—Apparatus for aerosol transmission of Newcastle disease virus in chickens.

The experimental chickens were of the New Hampshire breed, 6 to 8 weeks old, and free of Newcastle disease on the basis of clinical appearance and absence of hemagglutination-inhibition activity. The birds were reared in a building used only for normal chickens. After exposure, the birds were housed in groups of three in small Horsfall-Bauer units in an isolation building.

The aerosol apparatus, shown in figure 1, consisted of a number 40 De Vilbiss Nebulizer with an attached muzzle consisting of a large glass funnel covered with a rubber membrane having a slit aperture. The performance of this nebulizer has been extensively studied with various infectious agents.[7] It produces a fine cloud with tryptone broth suspensions of Newcastle disease virus. The critical particle size obtainable under the circumstances has not been evaluated. The effectiveness of the nebulizer with the closed muzzle attachment has not been studied critically. The consistent infection rates obtained in numerous trials with dilute virus suspensions indicate that it provides reasonably reproducible and infective virus aerosols in the chamber for short periods. According to trials, 60 pulsations of the rubber bulb during an interval of 1¼ minutes nebulized approximately 0.05 ml of the virus suspension. The beak of the chickens was held in the muzzle during the period of active nebulization.

PROCEDURE

Twelve birds were exposed to each strain of virus. The virus aerosol created in the funnel chamber with a 10^{-2} dilution of infected allantoic fluid in tryptone broth was breathed by each bird for a period of 1¼ minutes. The 12 birds were then separated into 4 cage groups of 3 birds each, hereafter designated A, B, C, and D. Groups A and B were sacrificed on the 4th day post exposure and separate tissues taken to deter-

7. Rosebury, T. 1947, Experimental Airborne Infection, Williams and Wilkins Co.

TABLE 1.—*Comparative data on tropisms of six strains of Newcastle disease virus as indicated by virus concentration in tissues, signs, lethality and postmortem findings.*

Newcastle strain	Neuropathogenicity				Pneumopathogenicity			Visceropathogenicity				Hemopathogenicity		Other effects		
	Brain titer embryo LD_{50}	Jerking of head and neck*	Leg paralysis*	Wing paralysis*	Lung titer embryo LD_{50}	Respiratory distress*	Cloudy air sac*	Trachea exudate*	Spleen titer embryo LD_{50}	Diarrhea*	Proventricular hemorrhage*	Intestinal hemorrhage*	Blood titer embryo LD_{50}	Days post exposure of viremia†	Embryo LD_{50} titer muscle	Lethality*	Intravenous LD_{50} chicken
CG	10^{-3}	0	6	6	10^{8}	0	4	1	10^{8}	1	3	2	10^{8}	2.3.4.	10^{-3}	6	10^{7}
GB	10^{-4}	4	5	6	10^{7}	1	5	2	10^{8}	4	4	3	10^{8}	2.3.4.		6	10^{7}
RO	10^{-4}	1	3	4	10^{7}	0	5	0	10^{8}	0	4	1	10^{8}	2.3.4.	10^{-4}	6	10^{8}
ROAK		0	0	0	10^{8}	0	3	0	10^{8}	0	2	0	10^{8}	2.3.4.	10^{8}	0	10^{1}
KM	10^{3}	2	0	0	10^{8}	0	3	0	10^{8}	0	2	1	10^{8}	2.3.4.		0	10^{1}
B1		0	0	0	10^{8}	0	1	0	10^{-4}	0	0	0		3.4.		0	10^{1}

* Number of birds out of a lot of 6 showing the signs or lesions described.
† Data collection terminated on 4th day.

mine the virus titer of each. Groups C and D were observed until death or the termination of the experiment.

The lungs, spleen, brain and breast muscle were removed aseptically from each of the 6 birds in groups A and B. Blood was obtained from the heart prior to sacrifice. Tissues were stored at −20 C until tested. In preparation for titration the lungs from the 3 birds of group A were minced separately and then mixed in approximately equal portions. One gram of the pooled minced lung was ground in a TenBroeck grinder with 9 ml of tryptone broth. The suspension was centrifuged at 2000 r.p.m. for 10 minutes. The supernatant fluid was designated as a 1:10 dilution of virus and served as the source of other dilutions. The several other tissues from group A birds and the lots of tissues from group B birds were prepared in the same manner.

An equal volume of an antibiotic solution composed of 5000 mg of streptomycin and 5000 units of penicillin in 1 ml was added to the inoculum to prevent bacterial growth. Virus dilutions of 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7} mixed with the antibiotic were inoculated in 0.2 ml amounts in 10-day embryonating eggs. Each dilution was injected into the allantoic chamber of 4 eggs. The eggs were candled twice a day and incubated until death of the embryo or for one week at 36 to 37 C. Specific virus deaths were considered to be of those embryos which died 2 to 7 days after inoculation, and whose allantoic fluids possessed hamagglutinating activity. The tissue suspensions and the allantoic fluids were cultured for bacterial contamination on nutrient agar slants and thioglycolate medium.

RESULTS

The titers of the lung, spleen, brain, breast muscle, and blood of the several virus strains (CG, GB, R0, Roakin, KM and B1) as determined by duplicate tests are recorded in figures 2 and 3. Comparisons of tissues titers with symptoms and gross pathologic changes can be made from the data in table 1.

The virus of all six strains multiplied to high titer in the lung, and in every instance the titer was higher in this organ than in other tissues. The nonlethal strains produced slightly lower titers in the lung than did the lethal strains. B1, which was the least pathogenic for chickens, developed only 1/1000 as

much demonstrable virus in the lungs as was produced by GB. However, the differences between some pathogenic strains and some strains of low pathogenicity probably were not significant. The occurrence of air sac involvement was greatest among those chickens whose lungs demonstrated the highest titers of virus.

The virus titers of the brain of infected chickens were closely correlated with the presence or absence of nervous symptoms, virus not being detected in the brain of chickens which did not manifest nervous symptoms. While presence of virus in the brain even in moderately high titer did not indicate that the strain producing the infection was lethal for the chicken, lethal strains invariably invaded the brain although sometimes achieving only low titers. Furthermore, only one of the three nonlethal strains invaded either the central nervous system or the spleen. Leg or wing paralysis, or both, were the most common symptoms of neural involvement. A spasmodic jerking of the head and neck was produced by two strains. Torticollis did not appear in any of the test birds. The temperature of some birds infected with GB strain dropped to as low as 35 C prior to death.

All three of the lethal strains and one of the nonlethal strains invaded the spleen, producing titers in that organ which ranged from 10^2 to 10^6 embryo LD_{50}. Proventricular lesions, often mild, were seen in all groups of birds receiving strains that developed a demonstrable concentration of virus in the spleen. Occasionally, diarrhea and hemorrhagic intestinal lesions were seen in birds affected with the lethal strains.

Viremia followed infection with all six strains of the virus. Viremia persisted longer and the virus was present in higher titers in chickens infected with lethal strains than in those inoculated

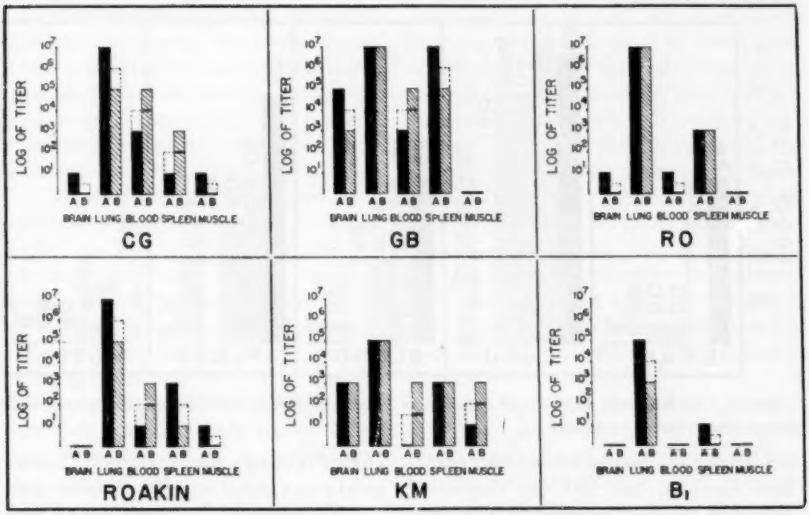

FIG. 2.—Tropism of 6 strains of Newcastle disease virus in chickens sacrificed 96 hours after infection by aerosol.

with the nonlethal ones.

Virus was present irregularly and infrequently in breast muscle regardless of whether the infection was produced by lethal or nonlethal strains of virus.

DISCUSSION

Work in this laboratory and in others[5] suggests that the factors which ultimately decide the course of Newcastle disease infection include (1) the rate of virus multiplication, (2) the degree of ability of the virus to transgress critical host barriers, and (3) the degree of ability of the host to respond with protective antibodies to antigenic stimuli. This study was concerned primarily with the second factor, but consideration of tissue affinity alone without reference to other aspects of host-virus interaction gives a picture with little meaning.

Native resistance of the animal to a disease agent often increases with age of the animal. Sabin[8] has shown that although mice less than 3 weeks old are highly susceptible to central nervous system infection with vesicular stomatitis virus introduced intranasally, mice 5 to 8 weeks old are highly resistant to infection. Refractivity is dependent in this instance on the development of a barrier to the virus in the olfactory pathway to the brain. Nutritional factors have been shown to affect the development of the barrier. Virus introduced directly into the brain, thus circumventing the olfactory barrier, produces a lethal infection in older mice.

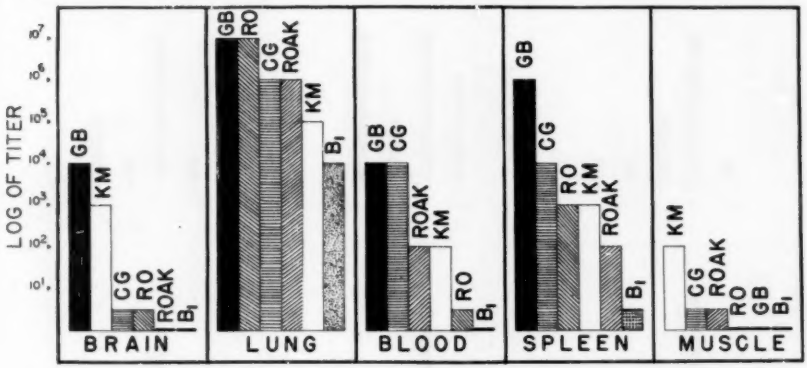

FIG. 3.—Variations in tropisms of 6 strains of Newcastle disease virus in chickens sacrificed 96 hours after infection by aerosol.

The pathogenicity of strains of Newcastle disease virus must always be determined in chickens of a specified age. Highly pathogenic strains kill a large percentage of adult chickens and all young chickens. Strains of intermediate pathogenicity kill few adult birds and a high percentage of young chickens. Strains of low pathogenicity kill few even of the very young chickens.

In the present study chickens of only one age group were used, and nutrition was maintained in a constant and adequate manner to reduce the variables as nearly as possible to differences between strains of virus. The differences known to occur in pathogenicity of

8. Sabin, A. B. and Olitsky, P. K. 1938, Influence of host factors on neuroinvasiveness of vesicular stomatitis virus. II. The effect of age and pathway of infection on the character and localization of lesions in central nervous system. J. Exper. Med. **67**: 201–228.

Newcastle disease virus strains for birds of several ages could depend upon differences in the ability of the strains to transgress certain host barriers. In 8-week-old birds, lethal strain GB invaded and multiplied in the brain and spleen while nonlethal strain B1 failed to invade either tissue. In this instance lethality appeared to be related to invasiveness. The relationship is not as clear when the neuroinvasiveness of three lethal strains of virus is compared with three nonlethal strains. While all lethal strains invaded the brain of part of the inoculated chickens by 96 hours, a longer period of study, as used by Karzon and Bang,[5] might have resulted in invasion of every individual. However, since one of the nonlethal strains, KM, invaded the brain and grew to a moderate titer, the passing of the brain barrier cannot by itself account for lethality. The other nonlethal strains, Roakin and B1, failed to invade the brain and to induce paralysis and death. B1 failed to produce paralysis or death when the virus was inoculated directly into the brain (unpublished data). This would suggest that this strain differs from the lethal ones in properties other than invasiveness.

Dissemination of the virus in the tissues of the host may be as closely related to growth rate of the virus and to the antibody response of the host as it is to the property of violating certain defensive barriers of the host. The slowness of the antibody response mechanism might fail, in the instances of strains multiplying more rapidly than others, to confine an infection which otherwise would be limited chiefly to the respiratory tract. The apparent tissue affinity or tropism of certain strains could be an expression of their growth rates.

Variability of the multiplication rate could be due to a variation in generation time (burst time) or to a variation in yield of virus particles from an infected cell (burst size). Either phenomenon would be favored by the accessibility to the more rapidly multiplying and more highly active cells of young chicken, or both could be affected adversely by introduction in the less active tissues of adult fowl. When tested in chicken embryos for which all strains were lethal, generation time was found to be identical for strains of high and low pathogenicity (unpublished data). The influence of age and species of host on generation time or burst size of the virus or the interrelationships of these factors to strain of virus and its tissue affinities remain to be established.

Most Newcastle disease virus strains appear to be highly antigenic. Within 6 to 7 days after infection antibodies may be demonstrated in the serum and, as antibodies appear, the virus disappears, although virus may be isolated from the brain and spleen at considerable intervals after the appearance of antibody in the blood (unpublished data).

Nonliving virus antigen can stimulate the production of demonstrable antibodies in 3 to 4 days—half the time usually required by live virus. Karzon and Bang[5] point out that antibody probably develops similarly in both situations but that the living virus binds the developing antibodies, with the result that it cannot be demonstrated until an excess occurs. Antibody would be highly important in modifying the course of disease, provided multiplication of virus was not too rapid or its distribution too general. Since chickens do not mature serologically until 6 weeks of age, they appear to be at a decided disadvantage in dealing with Newcastle disease virus prior to this time. This may account in part for the high lethality of the disease in younger birds.

SUMMARY

Six strains of Newcastle disease virus, some of high and some of low pathogenicity for chickens, have been studied quantitatively for their tissue predilection in chickens, following infection by aerosols of small quantities of virus.

The virus titers of the lung, brain, spleen, blood and breast muscle, as determined by duplicate trials, were compared with the signs and gross pathological changes of Newcastle disease. Appearance of certain signs and lesions was usually related to the invasion of certain organs by the virus.

The virus of all six strains multiplied to high titers in the lung. The blood contained the virus in five strain infections and the spleen in four strain infections. Four of the six strains were isolated from the brain. The two not found in the brain were the least pathogenic for chickens. However, one of the strains appearing in the brain in a titer of 10^3 embryo LD_{50} was not lethal to chickens. Two of the strains lethal to chickens were not isolated regularly from the brain. The third strain was found in high titer in the brain.

The three strains with the least pathogenicity for chickens were also least invasive.

Pathogenicity for chickens of Newcastle disease virus strains is discussed in relation to other characters of the strains.

ARIZONA PARACOLON RECOVERED FROM MIDDLE EAR DISCHARGE

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Members of the Arizona group of paracolon bacteria have frequently been recovered from reptiles and domestic animals but only on rare occasions from man. Of 456 cultures studied by Edwards, West and Bruner[1] in developing a workable scheme for the identification of different types, only 5 were obtained from human sources. Comparatively few additional isolations have been reported since that time.

It is possible that a more intensive search for members of this group would lead to more frequent isolations. In Georgia, two small outbreaks of gastroenteritis due to a paracolon of the Arizona group (Ar.10:1,2,5) were reported in 1950.[2] During the same year an identical strain was recovered from three sporadic cases of enteric disease.

To our knowledge, all previously isolated strains associated with human infections have been obtained from fecal material. It is the purpose of this paper to report the recovery of a member of the Arizona group from the ear of a patient with otitis media.

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The writers are indebted to Dr. Vilda Shuman, Waycross, Georgia, for providing the clinical data and to Dr. W. J. Murphy, Georgia Dept. of Public Health, Atlanta, Georgia, for assistance in preparation of the manuscript.

1. Edwards, P. R., West, M. G. and Bruner, D. W. 1947, J. Infect. Dis. **81**: 19.
2. Murphy, W. J. and Morris, J. F. 1950, J. Infect. Dis. **86**: 255.

FINDINGS

The patient, a Negro female, age 7, was admitted to the hospital on February 9, 1950 with suppurative drainage from the left ear, a rectal temperature of 105.2 F, and evidence of consolidation in the right lower chest. Subsequent studies showed that she also had sickle cell anemia. In spite of the free use of antibiotics, blood transfusions and dietary supplements, the patient ran a septic temperature which did not return to normal until the 33rd hospital day. On the 14th hospital day evidence of infection was observed in the right ear and three days later paracentesis was performed. The initial drainage was immediately cultured and from this material a paracolon was recovered.

Laboratory studies were not attempted until after antibiotics had been administered for some time. Blood cultures on February 27th and March 1st were negative. Stool cultures on March 16th, March 30th and April 14th were negative for known pathogens. Routine agglutinations were negative.

The broth culture obtained from the right ear on February 26th showed gram-negative rods and gram-positive cocci. Subcultures on blood agar and MacConkey's agar plates showed numerous colonies of a gram-negative rod and occasional colonies of *Staphylococcus albus* and diphtheroids.

On MacConkey's agar the colonies of gram-negative rods resembled those of shigella or salmonella. Subsequent biochemical reactions were similar to those obtained with members of the

Arizona group and the organisms were agglutinated by polyvalent antiserum. A culture forwarded to Dr. P. R. Edwards, Communicable Disease Center, Atlanta, Georgia, was identified by him as a member of the Arizona group of paracolon bacteria having the antigenic formula Ar. 5:13,14.

Blood specimens collected on February 15th and March 1st were checked for agglutinins against antigens prepared from the paracolon culture. With the serum obtained on February 15th, complete agglutination of the O and H antigens was observed in dilutions of 1:320 and 1:5280, respectively. Reactions with the serum collected March 1st showed little change in titer.

SUMMARY

1. A member of the Arizona group of paracolon bacteria was isolated from the ear of a child with otitis media.

2. O and H antigens prepared from the culture were agglutinated in high titer by the patient's serum.

3. This, apparently, is the first reported instance in which a member of this group has been recovered from human sources other than fecal matter.

COLORADO TICK FEVER

RECOVERY OF VIRUS FROM HUMAN CEREBROSPINAL FLUID

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Colorado tick fever is a disease without distinct neurological manifestations. However, a child with typical symptoms of this disease[1] had slight stiffness of the neck, and a lumbar puncture was done. The cerebrospinal fluid was normal and recovery from Colorado tick fever uneventful. The blood serum, from which the virus is always recovered, as well as the spinal fluid were inoculated into hamsters. Colorado tick fever virus was strongly suspected in the spinal fluid on the basis of white blood cell counts and typical spleen reactions in the inoculated hamsters.[1,2] Although viruses of various diseases have been found in the spinal fluid, it is not a common occurrence except in lymphocytic choriomeningitis.[3,4]

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We wish to thank Dr. Herald R. Cox and Dr. Hilary Koprowski and the Lederle Laboratories Division of the American Cyanamid Company, who generously supplied the antigen for the complement fixation tests.

This work was supported in part by funds supplied by James J. Waring, M.D. and the Continuing Research Fund of the University of Colorado School of Medicine.

1. Florio, L., Stewart, M. O. and Mugrage, E. R. 1944, J. Exper. Med. **80**: 165–188.
2. Black, W. C., Florio, L. and Stewart, M. O. 1947, Am. J. Path. **23**: 217–225.
3. Viral and Rickettsial Infections of Man, 1948, edited by Thomas M. Rivers, Lippincott Co.
4. Merritt, H. H. 1950, Bull. N. Y. Acad. Med. **26**: 547–554.

In order to determine if the virus of Colorado tick fever does occur in the spinal fluid, paired specimens of blood and spinal fluid were obtained from 6 other typical unselected cases. These were taken in all 3 phases of the disease from 5 children 2 to 8 years of age and a 31-year-old adult. Only 1 of these 6 individuals was suspected of having a stiff neck. In every instance, the spinal fluid pressure was normal and there were no chemical or cellular abnormalities. No red blood cells were observed in the spinal fluid. The various specimens were injected separately and carried in hamsters according to our usual procedure.[1] The animals were always inoculated initially with 1 ml of spinal fluid.

Pooled spinal fluid, obtained from 5 psychopathic patients, and serum from 8 animals selected at random from our colony were handled in the same manner. It is theoretically possible to introduce the agent into the spinal canal since the needle traverses presumably infected tissue. Therefore, two of these needles were immediately carefully washed in balanced salt solution and this material in each instance injected into one hamster and then passaged in the usual manner. These three groups are henceforth referred to as controls. The white blood cell counts are shown in table 1.

In all 7 cases, the white blood cell count on the hamsters was definitely low and indicative of the presence of virus in both the spinal fluid and serum. The original series of passages sometimes

TABLE 1.—*Mean white blood cell counts on hamsters inoculated with:*

(*a*) *paired serum and spinal fluid from 7 cases of Colorado tick fever*
(*b*) *washings from needles used in performing lumbar puncture on 2 cases of Colorado tick fever*
(*c*) *pooled spinal fluid from 5 psychopathic patients*
(*d*) *serum from 8 hamsters selected at random*

	Case no.	Number of animals	Number of passages	White blood cell count in thousands per mm³, mean and S.D.
a	1B*	25	9	4.57±0.47
	1S*	37	12	4.95±1.17
	2B	33	9	5.48±1.73
	2S	35	9	5.43±1.79
	3B	37	10	4.44±0.69
	3S	38	10	4.77±0.77
	4B	38	10	5.23±0.94
	4S	37	9	4.69±0.87
	5B	31	9	4.65±0.93
	5S	32	9	5.33±1.73
	6B	35	9	4.79±0.96
	6S	38	11	5.28±1.35
	7B	31	9	4.55±0.59
	7S	38	10	4.54±1.05
Total B		230		4.83±1.06
Total S		255		4.99±1.32
		Control groups		
b	3N*	35	10	7.60±1.27
	4N	36	10	7.43±1.12
c	PSF*	27	7	7.63±1.04
d	HS*	30	7	7.87±1.12

* B = blood serum, S = spinal fluid, N = needle washings, PSF = pooled spinal fluid, HS = hamster serum.

results in average white blood cell counts above 5000, since several hamster passages are often required in adapting a new strain of virus to the animals.[5] The washings from the needles used to do the lumbar punctures, the pooled control spinal fluid and the serum from the 8 animals in the colony gave normal white blood cell counts.[6]

Complement fixation tests were done on serum from hamsters inoculated with 4 of the blood and spinal fluid specimens, from various controls and from animals inoculated with 3 laboratory strains, according to the method of De Boer and Cox.[7] Their results with Colorado tick fever hyperimmunized mice were excellent.[8] Serums for these tests were taken at various times from the 4th day to 8 weeks following the original inoculation. All of these animals except those infected with the laboratory strains were eventually hyperimmunized by the use of homologous material in two to four 0.5 ml doses given one week apart. Since the results did not seem to depend on the time of bleeding, the data are combined in table 2.

Small groups of normal animals selected at random were bled and used as presumably negative controls for each group of tests. Four of these 30 serums fixed complement. Hamster and human serums that gave strongly positive results were also used as controls.

An examination of the results shows that the average white blood cell counts were in the infected range for the four paired serums and spinal fluids and three laboratory strains. If all positive results including the ± reactions are combined, 65 and 51%, respectively, of the tests done on the serum and spinal fluid from four cases of Colorado tick fever, and 33% of the tests on the three laboratory strains, fixed complement. The controls, which include washings from the needles, pooled spinal fluid and the normal hamster serum, all had normal white blood cell counts. However, 17% of these serums fixed complement. These results suggest that the virus of Colorado tick fever is to be found in the spinal fluid. They are not conclusive, however, since the laboratory strains of Colorado tick fever virus fixed complement poorly and the controls which should have been negative gave instead a significant per-

5. Florio, L., Miller, M. S. and Mugrage, E. R. 1950, J. Immunol. **64**: 257–263.
6. Stewart, M. O., Florio, L. and Mugrage, E. R. 1944, J. Exper. Med. **80**: 189–196.
7. De Boer, C. J. and Cox, H. R. 1947, J. Immunol. **55**: 193–204.
8. De Boer, C. J., Kunz, L. J., Koprowski, H. and Cox, H. R. 1947, Proc. Soc. Exper. Biol. & Med. **64**: 202–208.

TABLE 2.—*Results of complement fixation tests with hamster passaged:*

(a) Paired serum and spinal fluid from 4 cases of Colorado tick fever
(b) laboratory strains
(c) washings from needles used in performing lumbar punctures on 2 cases of Colorado tick fever
(d) pooled spinal fluid from 5 psychopathic patients
(e) serum from 8 hamsters selected at random

	Case no.	Number of animals with white blood cell counts‡	White blood cell count in thousands per mm³, mean and S.D. 4 days after original inoculation	Complement fixation reactions				
				Average of 1:4 and 1:8 dilutions		Negative	Anticomplementary	Total†
				4, 3, or 2	1, ±			
						Number of tests		
a	1B*	24	4.27±0.38	35	2	2	0	39
	1S*	24	4.35±0.46	9	21	28	0	58
	3B	24	4.46±0.42	13	10	13	0	36
	3S	25	4.61±0.60	10	4	23	0	37
	6B	18	4.18±0.29	26	23	50	4	103
	6S	18	4.54±0.82	22	24	62	1	109
	7B	23	4.45±0.69	19	7	4	0	30
	7S	25	4.37±1.29	21	13	6	0	40
Total B				93	42	69	4	208
Total S				62	62	119	1	244
			Laboratory strains§					
b	HI-1	16	4.63±0.61	8	2	14	0	24
	HI-2	5	4.68	0	0	18	0	18
	HI-3	16	4.81±0.73	6	5	12	0	23
			Control groups					
c	3N*	25	7.62±0.79	1	11	51	9	72
	4N	25	7.59±1.64	3	9	26	2	40
d	PSF*	25	7.10±0.88	0	6	42	33	81
e	HS*	31	7.67±0.51	8	3	31	4	46

* B = blood serum, S = spinal fluid, N = needle washings, PSF = pooled spinal fluid, HS = hamster serum.
† Some animals were bled more than once.
‡ Fifty animals were injected in each group except for HI-1, HI-2 and HI-3. The animals that had white blood cell counts represent random samples. All of the animals in the HI-1, HI-2 and HI-3 groups had white blood cell counts.
§ Established laboratory strains of Colorado tick fever virus. Original human isolations from naturally acquired cases.

TABLE 3.—*Neutralisations with hamster immune serum of presumed blood and spinal fluid strains of Colorado tick fever virus.*

Virus strains	Minimal infective dose, 8 animals each					Neutralization		
						Controls, 4 animals each	8 animals each	
							10 MID virus per animal +	
	10^{-3}	$10^{-3.3}$	10^{-4}	$10^{-4.3}$	10^{-5}	1 MID virus + 10^{-2} ml normal hamster serum	10^{-3} ml immune serum	10^{-4} ml immune serum
						Mean white blood cell count in thousands per mm³		
1B*			4.70†		8.00	4.53	7.66	4.68
1S*			4.61		8.06	4.75	7.29	4.38
2B		5.00		7.33†		4.10	7.81	4.21
2S		5.46		7.19		4.45	8.21	4.46
3B			4.43		7.74	4.20	7.50	4.19
3S			4.46		7.73	4.55	7.54	4.45
4B			4.90		7.73	4.98	7.70	4.81
4S		4.46		7.71		4.13	7.43	4.49
5B			4.53		7.28	4.18	7.99	4.48
5S			4.88		7.55	4.30	7.99	4.55
6B			4.73		7.73	4.35	7.35	4.16
6S	4.88		6.03			4.75	8.16	5.34
7B			4.43		6.94	4.20	8.11	4.21
7S			4.45		6.26	4.08	7.96	4.30‡
Mean and S.D.:								
Total B		4.67±0.37		7.54±0.93		4.36±0.41	7.73±0.96	4.39±0.40
Total S		4.74±0.97		7.13±1.63		4.43±0.38	7.80±1.00	4.58±0.62

* B = blood serum, S = spinal fluid.
† There were only 7 animals in these groups.
‡ There were only 6 animals in this group.

TABLE 4.—*Cross immunity between established laboratory strains of Colorado tick fever virus and blood and spinal fluid strains, with a challenge of the various control groups by established laboratory strains.*

Virus strains	Original inoculation: Number of animals†	Original inoculation: White blood cell count in thousands per mm³ mean and S.D.	Challenge: Virus strains	Challenge: Number of animals	Challenge: White blood cell count in thousands per mm³, mean and S.D.	Control: Number of animals	Control: White blood cell count in thousands per mm³, mean and S.D.
1B*	57	4.34±0.44	Laboratory strains‡	53	7.15±1.22	51	4.57±0.81
1S*	64	4.40±0.42	Laboratory strains‡	52	6.58±1.61	50	4.57±0.82
2B	23	4.51±0.45	Laboratory strains‡	17	7.59±1.23	30	4.81±0.95
2S	20	5.02±1.22	Laboratory strains‡	18	7.73±1.25	30	4.81±0.95
3B	51	4.29±0.43	Laboratory strains‡	46	7.57±0.77	35	4.38±0.42
3S	52	4.54±0.51	Laboratory strains‡	46	7.90±1.50	35	4.37±0.41
4B	36	4.68±0.48	Laboratory strains‡	32	7.64±0.80	46	4.70±0.80
4S	24	4.37±0.39	Laboratory strains‡	18	7.80±1.15	30	4.71±0.97
5B	27	4.39±0.58	Laboratory strains‡	18	7.77±1.42	28	4.45±0.35
5S	27	4.54±0.49	Laboratory strains‡	19	7.63±1.00	25	4.44±0.32
6B	45	4.32±0.35	Laboratory strains‡	52	7.66±0.99	42	4.43±0.39
6S	63	4.89±0.95	Laboratory strains‡	76	7.32±1.04	46	4.42±0.38
7B	47	4.35±0.55	Laboratory strains‡	43	8.22±1.75	30	4.56±0.46
7S	49	4.36±0.99	Laboratory strains‡	55	8.09±1.64	33	4.52±0.46
Total B	286	4.39±0.47		261	7.63±1.24		
Total S	299	4.57±0.78		284	7.50±1.47		
All controls						146§	4.49±0.60
			Laboratory strains‡				
HI-1	32	4.66±0.95	1-7B	10	7.81		
			1-7S	11	7.65		
HI-2	30	4.53±0.36	1-7B	9	7.45		
			1-7S	9	7.95		
WF-10	34	4.55±0.36	1-7B	10	7.85		
			1-7S	12	7.53		
LI-1	29	4.51±0.38	1-7B	7	7.44		
			1-7S	7	9.41		
Total	125	4.56±0.58	1-7B	36	7.66±0.88	28	4.38±0.44
			1-7S	39	8.00±2.06	28	4.40±0.36
			Control groups				
3N*	25	7.62±0.79	Laboratory strains‡	22	4.40±0.44		
4N	25	7.59±1.64	Laboratory strains‡	26	4.68±1.02		
PSF*	25	7.10±0.88	Laboratory strains‡	19	4.30±0.46		
HS*	25	7.58±0.51	Laboratory strains‡	20	4.70±0.36		
Total	100	7.47±1.07	Laboratory strains‡	87	4.53±0.68		

* B = blood serum, S = spinal fluid, N = needle washings, PSF = pooled spinal fluid, HS = hamster serum.
† Some groups were larger but white blood cell counts were done on random samples in these groups.
‡ Laboratory strains: HI-1 and HI-2 are original human isolations from naturally acquired Colorado tick fever, WF-10 represents an isolation from naturally infected wood ticks, LI-1 represents an isolation from naturally infected dog ticks from Long Island, N. Y.
§ Some animals were used as controls on several simultaneous experiments but are included only once in the total.

cent of positive results. Therefore it was decided to test this tentative conclusion by using neutralization and cross immunity experiments which are known to be effective techniques in identifying Colorado tick fever virus.[9]

Table 3 shows the minimal infective dose necessary to infect hamsters with passaged material from both blood and spinal fluid.[5] It also compares the blood and spinal fluid by measuring the amount of immune hamster serum that is necessary to neutralize the infectious agent.

The minimal infective dose for the blood and spinal fluid was identical for 5 of the strains. In the other 2 cases, the blood strains were more potent. The neutralization studies showed no difference between the blood and spinal fluid. In every instance, 10^{-3} ml of hamster immune serum neutralized 10 minimal infective doses of virus, whereas 10^{-4} ml of immune serum failed to do so.

9. Florio, L., Miller, M. S. and Mugrage, E. R. 1950, J. Immunol. **64**: 265–272.

Finally, cross immunity experiments were carried out as follows.[9] (1) Hamsters previously infected with the 7 blood and spinal fluid strains were challenged with 4 laboratory strains. (2) Hamsters previously infected with 4 laboratory strains were challenged with the 7 blood and spinal fluid strains. (3) The various control animal groups were challenged with laboratory strains. The results are shown in table 4.

The animals given the blood and spinal fluid strains were originally infected with 1 to 400 and occasionally 5000 minimal infective doses of virus. They were challenged 3 to 6 weeks later with 10 and occasionally 100 minimal infective doses of an established laboratory strain of virus. A comparison of results using various minimal infective doses followed by challenges with 10 or 100 doses of virus always gave identical results, and therefore the various groups were combined. The laboratory strains were always infected with 1 minimal infective dose and always challenged with 10 minimal infective doses of the blood and spinal fluid strains. The control groups were challenged with one minimal infective dose of the established laboratory strains.

In every instance, whether the blood and spinal fluid were used to challenge animals recovered from infection with our laboratory strains, or vice versa, the white blood cell counts on the challenged hamsters were in the normal range, showing them to be immune. Contrariwise, in every instance when the various control groups were challenged with our laboratory strains, the average white blood cell counts were in the infection range, indicating that they were not immune.

SUMMARY AND CONCLUSIONS

1. Spinal fluids and blood serums from seven individuals with typical Colorado tick fever were studied by the following techniques to determine if the virus of the disease could be found routinely in the spinal fluid, as it is in the blood serum:
   (a) white blood cell counts on serial hamster passages
   (b) complement fixation
   (c) comparison of minimal infective doses of blood and spinal fluid strains
   (d) neutralization
   (e) cross immunity; animals recovered from infection with laboratory strains were challenged with the experimental strains and vice versa

2. The controls consisted of hamsters injected with spinal needle washings, control spinal fluids and normal hamster serum. These were studied as follows:
   (a) white blood cell counts on serial hamster passages
   (b) complement fixation
   (c) cross immunity; these animals were challenged with one minimal infective dose of the established laboratory strains

3. The results are all consistent with the conclusion that the virus of Colorado tick fever is found routinely in the cerebrospinal fluid of individuals with this disease.

DIFFERENTIATION OF COXSACKIE VIRUSES BY ALTERING SUSCEPTIBILITY OF MICE WITH CORTISONE

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All of the known members of the Coxsackie family of viruses (C viruses) produce in suckling mice a systemic disease predominantly involving skeletal muscle. However, because of the accumulating evidence that the individual members of this group of viruses differ among themselves in many respects, it has become apparent that the currently designated family of Coxsackie viruses is a heterogeneous group that requires further classification. In addition to differing among themselves serologically (Dalldorf, 1950a and b; Melnick, 1950; Sulkin et al, 1950) and in their pathogenesis in experimental animals (Gifford and Dalldorf, 1951; Godman et al, 1952), differences in thermo-susceptibility (Robinson, 1950; Stanley, 1951), effect of pH (Robinson, 1950), size (Quigley, 1949; Himmelweit et al, 1950; Melnick et al, 1951) and probably even in epidemiologic patterns (Curnen et al, 1949; Shaw et al, 1950; Huebner et al, 1951; Lazarus et al, 1952) occur among the various types. Although increased susceptibility of animals treated with cortisone has been reported for a number of viruses (see Shwartzman and Fisher, 1952), only a slight increase has been noted in most cases. Since fatality or increase in titer of Coxsackie virus occurs rarely in normal adult mice, the lethal infection of cortisone-treated adult mice with two strains of C viruses reported by Kilbourne and Horsfall (1951) is significant. It seemed worthwhile, therefore, to extend these observations to other serologic types of C viruses to see whether differences might be found among the types that would allow further classification. This report concerns (a) the susceptibility of adult and weanling untreated and cortisone-treated mice to various types of C viruses inoculated by different routes, (b) serial passage and extent of adaptation of two types of C virus in cortisone-treated adult mice, and (c) weight change responses in mice inoculated with these viruses.

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MATERIALS AND METHODS

All C virus strains have been maintained by passage in 2-day-old Swiss albino mice. Three group A strains, Dalldorf type 1 (T.T.), Dalldorf type 2 (Fl.) and Dalldorf type 3 (J.Ol.), were supplied by Dr. Gilbert Dalldorf. The group A, type 4, Dallas-M.B. strain, which is serologically similar if not identical to the Texas-1 strain, was isolated at this laboratory (Sulkin et al, 1950). The Texas-1 strain and the three group B strains, type 1 (Conn. 5), type 2 (Ohio-R), and type 3 (Nancy), were provided by Dr. Joseph L. Melnick.

All virus suspensions used for inoculation consisted of brain or carcass tissue in distilled water with 2000 units of penicillin and 10 mg of streptomycin per ml. Supernatants following 2000 to 3000 r.p.m. for 10 to 15 minutes were used. Prior to animal inoculation suspensions were tested for bacterial contamination by culture on rabbit blood agar and thioglycollate mediums.

Swiss albino mice, Carworth Farms no. 1 strain, were used. Except where specified otherwise, all virus titrations were done in 2-day-old suckling mice and the Reed-Muench method was used to calculate end points. Infant mice dead or missing within 36 hours and adult mice dead within 48 hours after inoculation were not included in the data. The proliferation of C virus in

mice that died was ascertained by showing 10^{-3} suspensions of their hind limb or CNS tissues to be infective for infant mice.

Cortisone* was Merck brand "Cortone," which is described as a microcrystalline suspension of 25 mg of cortisone acetate per ml with added suspending agents and 1.5% of benzyl alcohol. Two and one-half to five mg of cortisone in 0.1 to 0.2 ml were not toxic to 7- and 15-g mice, respectively, in preliminary tests. The test animals received the cortisone in a single subcutaneous injection 1 to 2 hours before the virus inoculation.† Five mg of the drug were used for the 15-g adult mice and 2.5 mg were used for the 7-g weanling mice. The dose used for adult mice was the same as that used by Kilbourne and Horsfall (1951).

infected suckling mouse tissue was inoculated intraperitoneally or intranasally into cortisone-treated and untreated 7- and 15-g mice. The animals were examined twice daily during an observation period of 14 days for symptoms of C virus infection. Each variable was tested with at least one group of 10 mice, and the results represent in most cases three or more such groups of mice tested on separate occasions. Brain tissue in the case of group B strains and carcass for group A strains were harvested from all adult mice dying in the first group tested and their infectivity for suckling mice tested.

TABLE 1.—*Influence of cortisone, weight, and route of inoculation on susceptibility of mice to lethal infection with Coxsackie viruses.*

Coxsackie virus		Virus inoculum, LD_{50} dose	15 g mice		7 g mice	
Group	Type and strain		Intraperitoneal inoculation	Intranasal inoculation	Intraperitoneal inoculation	Intranasal inoculation
A	1 T.T.	10^8	−* / −	− / −	± / −	+ / −
	2 Fl.	10^8	− / −	± / −	++++ / +	+ / −
	3 J.Ol.	10^8	− / −	− / −	± / −	− / −
	4 Texas-1	10^6	± / −	± / −	++++† / +++	++† / −
	4 Dallas-M.B.	10^7	− / −	− / −	+ / ±	+ / −
B	1 Conn.-5	10^7	++++ / −	++ / −	++++† / +++	++++† / ++
	2 Ohio-R	10^6	− / −	± / −	++++† / −	++++† / ++
	3 Nancy	10^7	++++ / −	++++ / −	++++† / ±	++++† / ++

++++, 75 to 100% mortality; +++, 50 to 75%; ++, 25 to 50%; +, 1 to 25%; −, no deaths.
* The upper designation is for cortisone-treated mice, the lower for nontreated mice.
† Inoculum was 1000 times less than designated.

RESULTS

Susceptibility of 7- and 15-g cortisone-treated mice to certain serological types of C viruses.—A 10% suspension of virus-

The results of these experiments are summarized in table 1. The Conn.-5 strain fatally infects cortisone-treated 15-g mice in accordance with the report of Kilbourne and Horsfall (1951). This effect occurred with only one other serological type tested, the group B, type 3, Nancy strain. Following inoculation with the Ohio-R strain, most of the cortisone-treated adult mice became sick like those infected with the Conn.-5 and Nancy strains but recovered, and at the

* Obtained through the courtesy of Mr. A. W. Veazey of Merck and Co., Inc., Rahway, N. J.

† Limited numbers of experimental animals were tested using repeated doses of cortisone prior to viral inoculation, and no significant differences in susceptibility were manifested compared to the single administration of the drug used routinely in these experiments.

end of the 14 days were apparently healthy. No virus could be demonstrated in the tissues of these animals when sacrificed during the acute stage of illness. Less than 10% of cortisone-treated 15-g mice succumbed following intraperitoneal injection of the Texas-1 strain. No fatal injections were so induced with the other group A strains.

Seven-gram, cortisone-treated mice were more susceptible than such untreated mice to most of the strains tested. However, when a large proportion of cortisone-treated animals could be infected, it was with a strain of virus that caused at least an occasional death in untreated mice. The susceptibility of mice of this size to certain strains of C viruses has been shown previously (Dalldorf et al, 1949; Pappenheimer et al, 1951; Kunz et al, 1952; Goldblum, 1952). The all-or-none response evoked by cortisone with 15-g mice was, therefore, not seen with the 7-g mice. The group A, type 1 (T.T.), type 3 (J.Ol.) and type 4 (Dallas-M.B.) strains caused only rare infections of 7-g mice even when cortisone-treated. The quantitatively different results with the Texas-1 and Dallas-M.B. strains are striking since these are both group A, type 4 strains. That 7-g mice were more susceptible to the Texas-1 strain than to the Dallas-M.B. strain even without cortisone treatment cannot be accounted for on the basis of virus content of the inoculum. These strain differences are emphasized by the report that the Minnesota-1, type 4 strain causes infection of cortisone-treated adult mice (Graham et al, 1952), while the type 4 strains tested here did not infect such animals.

The route of inoculation seemed to have little effect on the result in cortisone-treated 15 g mice with the various strains of C virus used. The slight difference between the two routes observed with the Conn.-5 strain may be due to chance or to the erratic behavior to this strain often observed in these experimental animals. Following intranasal inoculation, all of the group B strains showed evidence* of pneumopathogenicity in the 7-g mice. The greater susceptibility by the intranasal route in nontreated mice to the Ohio-R and Nancy strains may be additional evidence for differential pathogenicity among strains. Under the conditions of the experiments no significant variation in susceptibility could be observed between the two routes of infection with group B strains in cortisone-treated 7-g mice. In contrast to the significant proportion of nontreated 7-g mice succumbing following intranasal inoculation with the group B strains, all such mice treated with the group A strains studied remained healthy. Moreover, only occasionally did cortisone-treated 7-g mice succumb following intranasal instillation of any of the group A strains tested.

Serial passage of C viruses in cortisone-treated adult mice.—Brain tissue was harvested from cortisone-treated 15-g mice dying of infection with the Conn.-5 and Nancy strains of C virus. One percent suspensions of these tissues were inoculated intraperitoneally into two groups of 15-g male mice, one group treated with cortisone and the other serving as controls. At least two brains were harvested of those dying in each cortisone-treated group, and a 1% suspension of these used for the next serial passage. Fifteen serial passages were made in this manner with the Nancy and Conn.-5 strains. Healthy animals from the control groups of each passage were sacrificed occasionally when animals in the cortisone-treated groups were acutely ill and dying. Injection of

* Histopathological studies were generously performed by Dr. Alice L. Smith.

tissue suspensions prepared from these animals into 2-day-old mice produced no infection. The identities of both the Conn.-5 and Nancy strains were established by specific neutralization in the fourth serial passage of each virus.

Comparative titrations were made in 2-day-old mice and in cortisone-treated 15-g mice with tissues harvested during the 1st, 5th, 10th, and 15th passages. The results of these titrations are summarized in figure 1, in which the virus titers of each strain in adult and suckling mice are plotted with virus passages. The increase in titer of the Conn.-5 strain for adult mice with a decrease in titer for suckling mice suggests adaptation to the former. In contrast to the case with the Conn.-5 strain after the fourth passage, the titer of the Nancy strain for suckling mice remained fairly constant as the titer for cortisone-treated adult mice increased. No tendency for either the Nancy or Conn.-5 strain to become adapted to 15-g mice not treated with cortisone, even on blind passage, was found. Such a result has been repeatedly obtained by other investigators (Gifford and Dalldorf, 1949). Considering that cortisone-treated 15-g mice occasionally died following inoculation with the Texas-1 strain, as shown in table 1, efforts to show proliferation of this virus were made by attempting serial passage in such mice. However, even serial blind passage was unsuccessful with this strain.

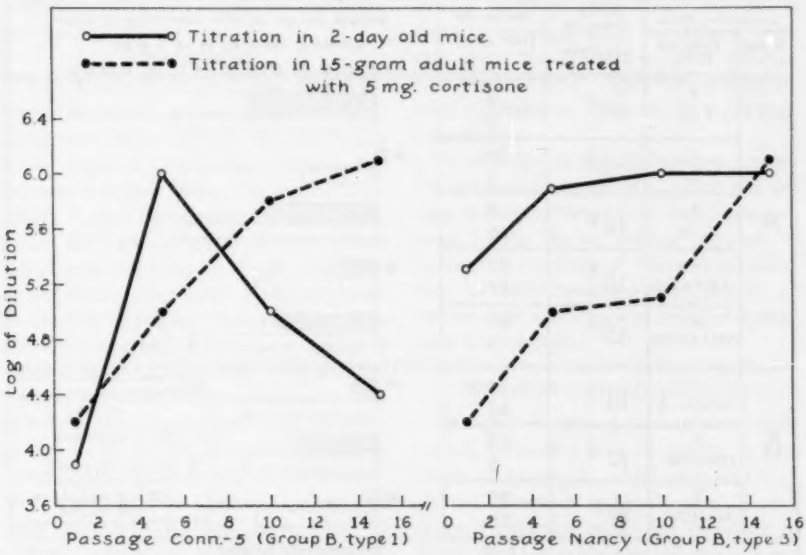

FIG. 1.—Comparative titrations in 2-day-old and adult mice of the Conn.-5 and Nancy strains of C virus following passage in cortisone-treated adult mice.

Average weights of mice following inoculation with C viruses.—Seven-gram mice to be inoculated intraperitoneally with the various serological types of C virus in the experiments described previously were weighed and the surviving animals again weighed

at the end of the observation period of 14 days. In the cases of the Conn.-5, Nancy, Dalldorf-2 and Texas-1 strains, where the survival of cortisone-treated mice was very low in the previous experiments, separate groups of such animals, which received a 10^{-4} virus suspension and 1.0 mg of cortisone per mouse, were tested. Several groups of uninoculated mice were observed for normal average weights. The average weights of the mice surviving in each group are given in figure 2.

Results appearing in figure 2 show that little or no weight gain occurs during the period of observation in cortisone-treated mice surviving inoculation with the Dalldorf-2 (Fl.), Texas-1, Conn.-5 and Nancy strains. Since a large proportion of animals in each of these groups succumbed, it may be assumed that nonfatal infection had occurred in many of these animals. On the basis that infection prevents normal weight gain, there may be little if any infection in mice not receiving cortisone when inoculated with the group A, type 1 (T.T.), type 3 (J.Ol.) and type 4 (Dallas-M.B.) strains, since terminal average weights of these were the same as with the uninoculated controls. However, cortisone-treated mice inoculated with these and the Ohio-R strains, and even nontreated mice inoculated with the Dalldorf 2 (Fl.), Texas-1, Conn.-5,

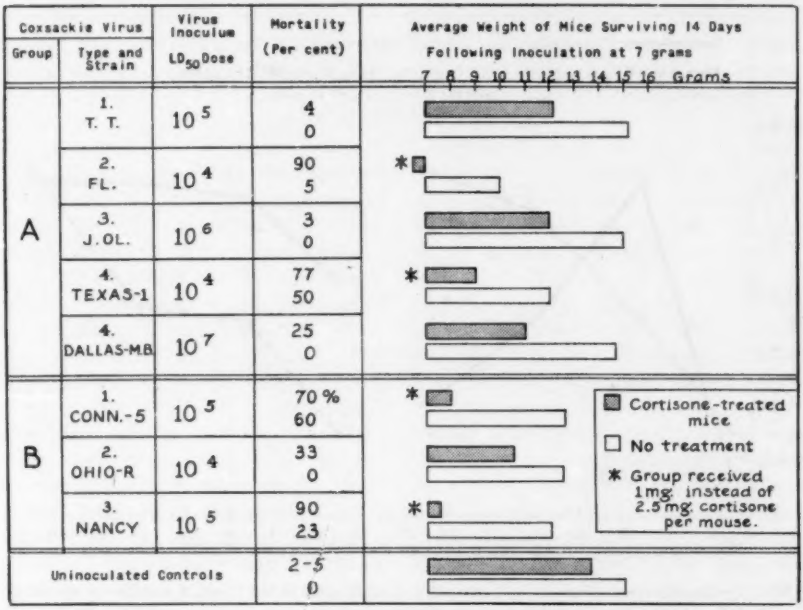

FIG. 2.—Average weights of mice surviving 14 days following virus inoculation.

Ohio-R and Nancy strains, did not show normal weight gain during the observation period, which may indicate nonfatal infections among these groups. Figure 2 may then indicate that with certain strains of C viruses mice which were 7 g at the time of inoculation and which survived suffered clinical infection and that the incidence of nonfatal infection shown by lack of normal weight gain is increased by cortisone treatment.

DISCUSSION

The evidence accumulates that individual members of the Coxsackie family of viruses differ among themselves in many respects. Of the eight strains of C viruses studied here, including four group A and three group B serological types, only two group B strains caused infections resulting in death of cortisone-treated 15-g mice. This was a reproducible biological characteristic with the strains tested. Two type 4, group A strains gave significantly different results in cortisone-treated 7-g mice and failed to produce a significant number of deaths in adult mice. On the other hand, fatal infection with the Minnesota-1 strain of this type 4, C virus has been reported (Graham, et al, 1952) in a large proportion of cortisone-treated adult mice. That strains of still another serological type differ is shown in the difficulties that Shaw (1952) as well as we ourselves (Donaldson et al, 1952) experienced in attempting to repeat the serial passage in embryonated eggs of the group A, type 2 virus originally reported by Huebner et al (1950). These results suggest that serological types cannot be classified on the basis of susceptibility of cortisone-treated mice.

Weller et al (1952) have reported that various cytopathological effects among C viruses could be seen in tissue culture. Moreover, differences have been observed in experiments on interference between various C viruses and Lansing poliomyelitis infections in mice (Sulkin and Manire, 1950; Dalldorf, 1951; Sulkin and Wallis, 1952). Determination of another significant characteristic has resulted from studies upon the effects of certain physical and chemical agents on these and other viruses. The group B, type 2, Ohio-R strain of C virus has been found to be susceptible to the effects of ethyl ether; its titer is greatly reduced after treatment with this agent for 2 hours at 37 C (Sulkin and Wallis, 1952). This did not occur with a number of other serological types tested.

Knowledge of the differences among the serological types of C viruses is accumulating to the extent that these are even being shown among strains of individual serological types. Classification of this heterogeneous family of viruses into more logical categories may soon be possible.

SUMMARY AND CONCLUSIONS

Of eight strains of Coxsackie viruses tested, including five of group A and three of group B, only two group B strains, Conn.-5 (type 1) and Nancy (type 3), caused infections resulting in death of 15-g mice treated with 5 mg of cortisone before inoculation. The susceptibility of 7-g mice to five of the eight strains tested was also increased. Three strains caused only rare infections of such mice even with cortisone-treatment. Difference between the two type 4, group A strains was observed in that one was infectious for 7-g mice and the other relatively not. While little difference was observed between the intranasal and intraperitoneal routes of infection in 15-g mice, intranasally inoculated 7-g mice were susceptible to the group B strains and relatively resistant to the group A strains even after cortisone-treatment.

Fifteen serial passages of two group B strains (Conn.-5 and Nancy) in cortisone-treated adult mice were accomplished without difficulty. Titrations of passage material showed that in each case the virus reached higher titers for cortisone-treated 15-g mice in the later passages than in the earlier ones, and the titer for suckling mice decreased with passage with the Conn.-5 strain. No tendency for either strain to become adapted to 15-g mice not treated with cortisone was found.

Observations of the weight gains during 14 days by mice that were inoculated at 7 g indicate that with certain strains of C viruses surviving mice have suffered clinical infection and that the incidence of nonfatal infection is increased by cortisone treatment.

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